A CORRELATIVE STUDY BETWEEN CERVICAL CYTOLOGY AND BIOPSY CERVIX

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CERTIFICATE

This is to certify that the dissertation entitled **A CORRELATIVE STUDY BETWEEN CERVICAL CYTOLOGY AND BIOPSY CERVIX** submitted by **Dr. G.VIMALA DEVI VIDYA** to the Faculty of Pathology, The Tamilnadu Dr. M.G.R. Medical university, Chennai in partial fulfilment of the requirement for the award of M.D. Degree in Pathology is a bonafide work carried out by her during the period June 2005 – Nov 2007 under my direct supervision and guidance.

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CONTENTS

INTRODUCTION

Carcinoma cervix is one of the leading causes of death of female population in developing countries. By virtue of its accessibility, cancer of cervix

can be readily diagnosed even in its preinvasive stage. If treated in the earlier stages the patient can often be cured of the disease.

For each case of cancer of body of the uterus, there are 25 cases of cancer cervix in India. A close quarters observation on the social behavior of our society reveals that most of the women have their marriages at very early part of their life leading to early age at first intercourse and poor sexual hygiene which are considered to be important etiological factors for cervical carcinoma.

George Papanicolaou M.D., Ph.D. after whom the Pap smear is named laid the foundation for preventive medicine at its best. He correctly estimated cytology as an infant science destined to become a titan. In the same way there were so many modifications made in the method of collection of specimen, techniques and methods of staining, interpretation and follow up.

The primary purpose of cervical cytology is to screen the population and identify the patients who have an abnormal pap smear. It has been the corner stone of cancer cervix screening for almost 50 years. Despite its enormous success as preventive screening tool for cancer cervix, the conventional pap smear has its limitations.

In the recent past, there has been emergence of various newer techniques to assess the proliferative capacity of cells. This has been an important criterion to assess the malignant behaviour of cells.

Various techniques are available like DNA content analysis, 'S' phase fraction calculation by means of DNA cytometry, proliferating antigens like C3, F10, DNA polymerase-2, Ki67 and PCNA. But these techniques are expensive and can be carried out in some sophisticated diagnostic research centers. A simple and inexpensive method is the staining and counting of the Nucleolar Organizer Regions. This is based on RNA transcription activity. Silver colloidal solutions of high concentration have been used for this purpose and this is called AgNOR stain. The number of AgNORS in a cell nucleus reflects the proliferative activity of the cell with progressive increase in number from normal cells to dysplastic and carcinomatous cells. AgNOR in cervical cytology has also been studied. AgNOR though expensive, the single step technique and ease, which it can be done, is very impressive. Pap though less expensive than AgNOR is a cumbersome procedure with lot of chemicals involved.

The definitive diagnosis of the patient's condition rests on the follow up histologic evidence from the biopsy material. Histologic diagnosis is frequently

used as a gold standard against which we calculate the accuracy of cytologic diagnosis.

Along with routine haemotoxylin and eosin staining in histopathological sections, AgNOR stain can be used. Studying the number, shape and distribution of AgNOR dots in the cell gives information not only about the morphology, but also about the behaviour of the cells. It is useful in differentiating doubtful cases of CIN.

AgNOR count also has prognostic significance. CIN lesions with low AgNOR counts are more likely to regress in comparison to CIN lesions with high AgNOR count. So it can be used as an adjunct to routine cytology and histopathology for diagnosis of cervical lesions in doubtful cases.

Hence the present study has been carried out with an attempt to correlate findings between cervical cytology and histopathological finding of biopsy cervix using Pap stain & AgNOR stain in cervical cytology and using H&E & AgNOR stain in histopathological sections.

AIM OF STUDY

- To evaluate Pap smears in patients attending the Gynecology department of Government Rajaji Hospital, Madurai.
- To study abnormal smears and subject the patient to biopsy study.
- To correlate the cytological findings with histopathological diagnosis.
- To assess the usefulness of the cytological study in the diagnosis of cervical lesions.
- To evaluate the role of Argyrophilic staining of nucleolar organizer regions in Pap smear and histopathological section of biopsy cervix.

REVIEW OF LITERATURE

ANATOMY:

The cervix is the lower portion of the uterus which connects this organ to the vagina through the cervical canal. It is divided into a portion that protrudes into the vagina called portio vaginalis, and that lies above the vaginal vault called supra vaginal portion. Outer surface of portio vaginalis is known as the exocervix or ectocervix and the portion related to the endocervical canal corresponds to endocervix. The lower opening of the endocervical canal is known as external os. The upper limit of the endocervical canal is known as internal os.

EMBRYOLOGY:

Cervix develops from the fused caudal portion of the Mullerian duct or Paramesonephric duct which is formed by the invagination and subsequent fusion of the coelomic epithelium.

The Epithelium of the Cervix is formed by the induction of basal cells from the underlying stroma which undergoes squamous differentiation. A portion of these cells remains uncommitted forming the reserve cells of the cervix. They are capable of both squamous and columnar cell differentiation.

HISTOLOGY:

The exocervix is lined by nonkeratinizing squamous epithelium and composed of three layers.

1. Basal cell layer
2. Midzone (Stratum spongiosum)
3. Superficial layer.

The Endocervix is lined by tall mucin secreting columnar epithelium that is highly branched and form endocervical glands that extend at an oblique angle to the cervical canal into the lamina propria.

The junction between squamous and columnar epithelium is known as squamo columnar junction. The position of the junction is variable due to both the cervical anatomy and the distribution of the basal and subcolumnar reserve cells that exist just cephalad of this junction. It is the progressive differentiation of these basal / reserve cells that governs the micro anatomy of this region, ultimately resulting in the cephalad migration of the squamo columnar junction.

The portion of the columnar epithelium that is ultimately replaced by squamous epithelium is termed the transformation zone. This is the site where precancerous lesions and squamous carcinomas develop. Koss identified that

more than 90 percent of intraepithelial lesions develop in the area of squamo columnar junction[1]. The remaining 10% of lesions are thought to originate from columnar epithelium. The connective tissue in the lamina propria of the cervix is more fibrous with blood vessels and nerves . The smooth muscles of the muscularis extend into the cervix, but are not compact.

HISTORY AND MODIFICATIONS IN PAP SMEAR

While studying the hormonal response of the human vaginal mucosa, George Papanicolaou, MD, Ph.D discovered that tumor cells could be found in vaginal fluid of women with cervical cancer[2].

Papanicolaou presented his paper entitled "New Cancer Diagnosis" at the Third Race Betterment Conference in Battle Creek, Michigan, in 1928[3]. Unfortunately, it reached only a limited audience and came at a time when histologic techniques were being perfected and cytologic methods were on the wane.

Furthermore, Aurel Babès, a Romanian pathologist, published a paper entitled "The possibility of diagnosing uterine cancer by the smear technic" in the Proceedings of the Conference of the Gynecologic Society of Bucharest on January 23, 1927[4].

Babes elaborated on this paper in April 1928 and clearly stated that his method was applicable to early cancers that had not yet penetrated the stroma.

Later, Babès made the statement that Kermauner and Schiller had used a modified vaginal smear method for the diagnosis of cervical cancer on a large scale with very good results[5].

Yet ironically the feeling at that time (as expressed by prominent oncologist James Ewing) was that since the uterine cervix was accessible to biopsy, the use of a cytologic examination was superfluous.

In 1943, the technique of diagnosing uterine carcinoma by cytology as well as the possibility of diagnosing early cervical cancer was accepted[3].

The concepts of early cancer and carcinoma in situ were widely understood and the potential of the 'Pap smear' for cancer prevention was finally appreciated. It was actually a Canadian physician, J. Ernest Ayre who described the method we know today as the Pap smear in the mid-1940s [6].

Papanicolaou had studied vaginal pool secretions easy to obtain, but tedious to screen. Ayre used a spatula instead the 'Ayre spatula' to directly scrape cells from the cervix[7].

In 1943 J.Ernest Ayre et al, emphasized the use of cervical os aspiration as a preferable technique for the diagnosis of uterine cancer and published 'A simple office test for uterine cancer diagnoses. Cervical os aspiration was superior to vaginal aspiration because a greater concentration of malignant cells was found in the direct cervical smear, both in clinical and preclinical lesions. In 1943 Papanicolaou and Marchetti used Cary's spatula or glass syringe and took endocervical and endometrial smear and studied. In 1947, Ayre et al used surface biopsy cell scrapping technique with Ayre's spatula[7].

A cervical smear or liquid-based cytology test is the routine test for detecting early changes in the cells of the cervix.

THE BETHESDA SYSTEM

Terminology used in cervico / vaginal cytology has evolved over the course of years. Papanicolaou's original system had five classes of increasing atypia. This system did not reflect the correct understanding of cervical neoplasia and did not have an equivalent in tissues diagnostic terminology. So it was eventually abandoned. It was progressively replaced by CIN systems in 1970. In 1988, National cancer institute (NCI) working groups made uniform descriptive terminology for cervical cytology and named as 'The Bethesda System (TBS)'. The 1988 Bethesda system of reporting cervix/vaginal cytology diagnoses

divided squamous intra epithelial lesions into the following categories.

1. Atypical squamous cells of undetermined significance (ASCUS)
2. Low Grade SIL
3. High Grade SIL
4. Squamous cell carcinoma

The main criticism of this system was that it leads to over treatment of many individuals whose smears were placed in Low Grade SIL category. So minor modifications were incorporated in 1991 Bethesda system which streamlined the terminology[8]. In addition, an adhoc committee developed criteria for specimen adequacy and diagnostic terms culminating in a TBS atlas that outlines and illustrates the morphologic feature[9]. The Bethesda System (2001) is currently followed.

The structure of The Bethesda System includes three factors namely,

a. Statement of specimen adequacy,
b. General categorization
c. Diagnostic terminology

A. SPECIMEN ADEQUACY

An adequate specimen is described in positive terms as one that is properly labeled, accompanied by relevant clinical information, appropriately

fixed and on microscopic examination demonstrates an adequate number of well preserved, evenly distributed evaluable cells reflecting an ectocervical and endocervical component[10].

Well preserved and well visualized squamous epithelial cells should cover more than 10% of the slide surface. An adequate endocervical / transformation zone component should at a minimum consist of two clusters of well preserved endocervical and / or squamous metaplastic cells. Each cluster should be composed of at least five cells[11].

B. GENERAL CATEGORIZATION

1. NEGATIVE FOR INTRAEPITHELIAL LESION OR MALIGNANCY

- Infections: Trichomonas vaginalis, fungal organisms like candida, Actinomyces, Cellular changes associated with Herpes simplex virus.
- Reactive cellular changes.

The epithelia covering the uterine ectocervix and endocervical canal are under the constant influence of physiologic stimuli but also of ever changing external stimuli[12].

Regenerative epithelial reactions are commonly found in patients after partial or complete destruction of epithelia by infection and inflammation.

Repair epithelium in experimental animals has been found to be more susceptible to the action of carcinogenic agents than non traumatized tissues[13].

Regeneration of cells as a manifestation of a reparative change can occur in squamous epithelium, squamous metaplastic epithelium and columnar epithelium[14]. Reparative reactions are frequent in patients who have had recurrent cervicitis and in patients who have had recent treatment such as punch biopsies, conization, cryosurgery, laser therapy and endocervical curettage This type of reaction is also found in cases of erosion cervix or ulceration of cervical stroma caused by prolapsed uterus or by an IUCD.

In cervical smear, cells from reparative epithelium appear as sheet like aggregates with indistinct cytoplasmic boundaries. The cells have a wide variation in size and shape. The cytoplasm is usually cyanophilic and sometimes finely vacuolated or may contain large vacuoles. Nuclei are mostly round to oval with some nuclear enlargement and variation in nuclear size. Nucleoli are prominent and multiple macronucleoli are sometimes present. The nuclear chromatin is finely granular, almost always evenly distributed and not hyperchromatic. These nuclear changes are due to active protein synthesis in the fast growing cells which try to replace the damaged epithelial cells.

The predominant arrangement of cells in sheet like aggregates and normochromic, finely granular, evenly distributed chromatin and the presence of macronucleli differentiate the cells from reparative changes and cells from invasive neoplastic processes. Some times, it may be difficult to differentiate between both of them. In such cases, a follow up smear after treatment of infection will be useful in which the above changes will reverse back to normal study.

Inflammation causes minor cytologic abnormalities such as dual reaction, lysis or vacuolation of cytoplasm, slightly dispropotionate nuclear enlargement and an increase in nuclear-cytoplasmic ratio. Regenerative changes of nuclei such as folding of nuclear membrane, karyorrhexis, karyolysis and pyknosis can also be seen.

Condylomatous lesions and epithelial abnormalities

It has been recognized that certain intraepithelial neoplastic lesions of the human cervix first defined as koilocytotic warty atypia by Koss and Durfee would now be diagnosed as condylomatous lesions caused by an infection with HPV[15]. HPV in particular the subspecies 6, 11, 16, 18, 31 and 35 were thought to be important sexually transmitted factors in the genesis of cervical cancer[16]. HPV types 6 and 11 are commonly associated with warty condyloma, flat condyloma

and low grade dysplasia (CIN Grade 1) whereas HPV types 6, 18, 31 and 35 are often found in high grade lesions of CIN.

HPV affects both the nucleus and the cytoplasm of the infected cells .It causes peripheral condensation of the cytoplasm of these cells giving a wire loop appearance. The squamous angular forms tend to become rounded and the cell assumes a softer, rounded or ovoid appearance. The nucleus is most often eccentrically placed with para nuclear halo in the intra cytoplasmic space. This type of cell is called Koilocyte. It has a high degree of specificity. Nearly 90% of cases of condyloma with koilocytic change were found to have demonstrable HPV antigen by immuno enzymatic technique.

2. EPITHELIAL ABNORMALITES

ASCUS (ATYPICAL SQUAMOUS CELLS OF UNDERTERMINED SIGNIFICANCE)

ASCUS reflects the reality and limitations of light microscopy in classifying cytologic changes. It should be used sparingly when morphologic changes exceed the parameters of a benign reactive process but are insufficient for a definitive diagnosis of a malignant lesion.

There is nuclear enlargement of squamous cells with mature superficial/ intermediate type cytoplasm. Nuclear enlargement is two and a half to three times that of a normal intermediate squamous nucleus, but the chromatin remains evenly distributed without significant hyperchromasia. Nuclear outlines are smooth and regular.

Atypical squamous cells associated with atrophy should be considered if cells show nuclear enlargement with concomitant hyperchromasia, marked irregularities in nuclear contour or chromatin distribution or marked pleomorphism in the form of tadpole or spindle cells.

Atypical squamous cells with metaplastic cytoplasm showing nuclear enlargement about one and half to two times normal raises the differential diagnosis of reactive metaplasia versus HSIL. In low risk population, the rate of ASCUS should be less than 5%. In high risk population the rate of ASCUS may be higher, but should not exceed two to three times the rate of SIL.

Correlation of ASCUS cases with the results of biopsy, correlation of ASCUS cases with HPV typing, review of ASCUS cases by a second cytopathologist and calculation of ASCUS / SIL ratio are the various quality assurance monitors for ASCUS. In 25% to 60 % of patients with ASCUS, however further evaluation will detect a squamous intraepithelial lesion[17].

SQUAMOUS INTRA EPITHELIAL LESIONS (SIL)

In TBS, low grade squamous intraepithelial lesion (LSIL) and high grade squamous intra epithelial lesion (HSIL) encompass the spectrum of precursors to squamous carcinoma of the cervix. LSIL incorporates changes of human papilomavirus (HPV) as well as mild dysplasia/CIN I[18]. HSIL includes moderate dysplasia /CIN2, severe dysplasia /CIN3 and carcinoma insitu / CIN3.

Diagnosis of LSIL based on cellular changes associated with HPV requires nuclear as well as cytoplasmic abnormalities. Nuclear changes may include enlargement with hyperchromasia, a pyknosis with chromatin smudging and wrinkling of nuclear contours. Cytoplasmic changes consist of a well defined perinuclear cavity, associated with peripheral thickening of the cytoplasm or cytoplasmic orangeophilia and rounding of cellular contours. LSIL changes typically involve 'mature' intermediate or superficial type cytoplasm with well defined polygonal borders[19].

Features that favour a high grade lesion include increased numbers of abnormal cells, higher nuclear to cytoplasmic ratios, greater irregularities in the outline of the nuclear envelope and nuclear chromatin distribution and increased number of chromocentres. The cells of HSIL have a more immature type of cytoplasm either delicate or lacy with rounded cell border.

This two tiered LSIL / HSIL approach attempts to morphologically distinguish minor from significant lesions.

MILD DYSPLASIA (CIN I OR LOW GRADE SIL)

CYTOLOGY

Cells from mildly atypical lesions such as mild dysplasia (CIN grade 1) usually have plentiful clear translucent cytoplasm with well-defined angular borders. Cells resemble intermediate and superficial type squamous cells with a somewhat reduced cytoplasmic body and a slightly enlarged nucleus, occupying less than one third of the total area of the cell. Nuclear chromatin is finely granular, evenly distributed and only slightly hyperchromatic[20].

HISTOLOGY

The upper two thirds of the epithelium usually show a relatively regular arrangement of cells, with preserved stratification. These layers are composed of cells recognizable as intermediate type and superficial squamous type cells with slightly reduced cytoplasmic volume and slightly increased nuclear size. Aberrations of nuclear morphology are predominantly limited to the most basal layers of the epithelium.

MODERATE DYSPLASIA (CIN II OR HIGH GRADE SIL)

CYTOLOGY

Most cells are round to oval, but spindle cells and elongated and bizarre shapes may occasionally be found. Cytoplasmic staining is cyanophilic, but a relatively high number of the cells may show eosinophilia of the cytoplasm. Nuclei are enlarged and round to oval, sometimes elongated or irregularly shaped. Nuclear chromatin is evenly distributed and slightly too moderately hyperchromatic. Nucleoli are usually absent. The nuclear/cytoplasmic ratio is increased[21].

HISTOLOGY

Only the upper one third of the epithelium still shows evidence of stratification of cells. Cell arrangement is disturbed in as much as two thirds of the thickness of the epithelium.

SEVERE DYSPLASIA (CIN GRADE III OR HIGH GRADE SIL)

CYTOLOGY

The size of the cells in severely abnormal intraepithelial changes is comparable with the parabasal cell type. Cytoplasm is usually sparse, typically forming a small rim around the nucleus. Cells are seen singly as well as in aggregates. The nucleus usually occupies at least two thirds of the total

area of the cell. Nuclei have a hyperchromatic irregularly distributed, coarsely granular chromatin. Some severe dysplasias show an extreme irregularity in shape and size of the composing cells.

HISTOLOGY

In severe dysplasia, cells show a greatly disturbed arrangement in all the three layers of the epithelium. Throughout the entire epithelium, cells show reduced maturation with loss of cytoplasmic volume and an increased nuclear size and shape and often have irregular forms.

CARCINOMA IN SITU

Carcinoma in situ is used to describe a lesion replacing the normal surface epithelium or the epithelium of the invaginations of the surface epithelium or both, in which all the layers of the epithelium are composed of abnormal poorly differentiated or largely undifferentiated cells[22].

CYTOLOGY

Cells occur singly or as syncytial aggregates with indistinct cell borders. Cells have only a minimal amount of cytoplasm. Nuclei vary in size and shape and frequently overlap. Nuclear chromatin is irregularly distributed and coarsely granular.

HISTOLOGY

Irregular arrangement of cells and almost complete lack of maturation. Most superficial layers show parakeratosis. Mitosis can be seen at all levels with absence of invasion.

INVASIVE CANCER OF THE UTERINE CERVIX

CYTOLOGY

Cells are often arranged in syncytial masses in which cells have indistinct boundaries. Round or oval forms are more numerous. Large round and oval nuclei with slightly irregularly distributed nuclear chromatin and irregular macronucleoli. Cytoplasmic eosinophilia is more often observed.

ATYPICAL ENDOCERVICAL CELLS OF UNDETERMINED SIGNIFICANCE

This category includes a broad morphologic spectrum ranging from atypical appearing reactive processes all the way to adenocarcinoma in situ (AIS).Therefore lesions falling into this category should be further subclassified, if possible, according to whether a reactive or a premalignant / malignant process (AIS) is favored.

ATYPICAL ENDOCERVICAL CELLS, PROBABLY REACTIVE

These cells demonstrate nuclear enlargement three to five time's normal and slight hyperchromasia. A honeycombed pattern with distinct cell borders is often maintained.

ATYPICAL ENDOCERVICAL CELLS, PROBABLY AIS

These cells are characterized by cellular strips and rosettes demonstrating elongated, overlapping nuclei with moderately coarse chromatin and hyperchromasia. The peripheral border of the glandular clusters may be 'feathered' with protruding nuclei, in contrast to the smooth communal border typical of glandular fragments.

ADVANCES IN SPECIMEN COLLECTION AND INTERPRETATION: SPECIMEN COLLECTION

Attempts to optimize the specimen itself for screening and interpretation have been made. The thin prep (cytyc, Marlborough) method has been introduced to improve the quality of specimen collection. Specimens are placed in a collection fluid that homogenizes and rinses the cells. The sample suspension is then used to create a series of thin prep slides, with cells concentrated in a $20mm^2$ areas. This method tends to optimize cell preservation and reduce artifacts that hinder interpretation. In one study of 251 patients, excellent

correlation between the standard Pap smear and the thin prep was noted[23]. Slides could be read faster, from a smaller sample area, and with fewer numbers of cells needed to make the correct diagnosis.

Computerized screening systems have been created[24]. PAPNET is a neural network computer processing system that screens conventionally prepared slides. The computer is programmed in pattern recognition and is able to identify abnormalities based on morphologic characteristics .From the computer review, selected abnormal cells are shown in 128 video images, 400x magnified fields with their corresponding grid location from the slide. The images are then reviewed by a cytologist to interpret the finding on the screen. Areas of concern on review are then confirmed by examination of the actual slide.

PAPNET system could retrospectively identify intraepithelial lesions missed on routine cytology from 19 to 20 patients who developed biopsy proven, high-grade or invasive lesions. Others have reported a 2.2% false negative rate reduction with PAPNET in a series of 638 smears.

ADJUVANT TESTS

A variety of adjuvant tests have been introduced to improve the

sensitivity and specificity of cervical cancer screening and to provide prognostic information. These tests include HPV detection, new techniques for visualizing acetic-acid –stained cervical specimens, and molecular markers. The association of HPV types 16,18,31, and 35 with high-grade dysplasia and cancer has led to HPV tests. With improvements in technology, HPV testing has become widely available. It has been used both in primary screening and in triaging patients into risk groups.

Molecular markers are being developed for the detection of cervical dysplasia or prediction of progression. Different molecular markers are expressed in normal, premalignant and malignant tissues. One such marker, the MN antigen is a protein expressed in dysplastic and malignant cervical tissue[25].

AGNOR STUDIES

Cell kinetics plays an important role in tumour behavior. Proliferation rates of the tumour can be assessed to determine the behavior of a particular tumor. The cell cycle can be divided into four phases based on the nuclear chromatin activity. They are, S.G1, G2 and G0 phases. The cells show active reduplication of DNA in the S Phase. G2 is the second resting phase before active mitosis. Thus the DNA content at the end of "S" phase is an indicator of proliferative activity. AgNOR detects the DNA content at this stage.

Stable cells are quiescent cells in G0 Phase and can be stimulated into G1 by an appropriate stimulus. The tumour suppressor gene p53 blocks the progression of cells through the cell cycle late in the G1 phase of replication. Mutant forms do not have this effect.

During the phase of active DNA replication, strips of DNA containing RNA genes are seen inside the nucleolus. These DNA fragments are actively transcribing with the help of polymerase 1 enzyme. These are considered as ribosomal factories. Cell cycle is controlled by a few enzymes called M Phase promoting factors.

The precise localization of NOR, has been found to be in the 18S DNA gene clutter in the stalk region. However, exceptions do occur.

An increase in the number of AgNORs counted in any given section could be explained as follows:

1. Increase in the number of AgNOR bearing chromosomes in the karyotype.
2. Increase in transcriptional activity producing more visible argyrophilic cells.
3. Increase in the proliferating rate of cells resulting in large population showing positivity.

4. Tumours with increased AgNOR counts in interphase nuclei are more likely attributable to cellular proliferation than to N ploidy because diploid cells are tetraploid in G2 phase transiently, resulting in temporary doubling of NOR bearing acrocentric chromosomes as found by Crocker et al (1989)[26] Immediately before and after mitotic division, the NORs disperse and then re-aggregate leading to increase in the number of countable AgNORs in the nuclei.

John Crocker et al, in 1990 enumerated standardized approach in counting AgNOR. Firstly all silver stained structures could be counted but when lying in groups each cluster (almost aggregated or partly disaggregated nucleoli) treated as one structure. Secondly, where AgNOR could be counted within a nucleolus each AgNOR could be counted as unit together with the smaller AgNOR seen outside the nucleus over all he suggest that total AgNOR dots both intra and extra nucleolar be enumerated[26].

In 1995 D. Prathiba et al studied the value of AgNORs in premalignant and malignant lesions of the cervix. The mean AgNOR count found to increase progressively from normal to CIN I, II, III, and invasive carcinoma[27].

In 1997, Calore et al conducted a study of organizer nucleolar regions by the argyrophil technique in cervical intra epithelial neoplasms and found that

AgNOR counting can be useful in the identification and classification of individual cases of intraepithelial neoplasia and their differentiation from eventual difficult cases of cervicitis[28].

In 1997, Jyotima Agarwal et al found that mean AgNOR was higher in CIN and Malignant lesions, when compared to squamous metaplasia and chronic cervicitis. And also adenocarcinomas have higher AgNOR when compared to squamous and adenosquamous carcinoma[29].

In 1998, Terlikowski S et al compared AgNOR counts of CIN III and SCC and suggested that AgNOR counts could be of significance in the evaluation of cervical lesions and could elaborate histopathological diagnosis[30].

In 2001 J.S. Misra et al performed an assessment of AgNOR count as tumor marker in cervical carcinogenesis in cervical smears. The statistical analysis revealed significant variation between normal and inflammatory smears. There was highly significant variation between inflammatory, LSIL, HSIL and frank malignancy smears[31].

In 1999, Kurian et al studied the relation between cervical glandular intraepithelial neoplasia to micro invasive and invasive adenocarcinoma of the

uterine cervix[32].

In 2001, Sakai Y et al had done morphometric evaluation of nucleolar organizer regions of intra epithelial neoplasia. They found that number of cells with one dot decreased with increasing CIN and the number of cells with more dots increased with increasing CIN. Total number of dots per 100 cells increased with increasing CIN and concluded that counting cells with 4 or more dots is the parameter for distinguishing the grade of CIN[33].

In 2003, Rajini Kawshik et al found AgNOR as a reliable indicator of cell proliferation and malignant potential of the lesion and can be used as an adjuvant in routine histopathology in cervical lesions[34].

ADVANTAGES OF PAP SMEAR

- No injury to tissue is produced allowing frequent sampling to know the progress of the disease or regression of the lesion
- Smears cover a wider surface area than that involved in a biopsy.
- Intimate cellular details are more often clearly seen in an isolated cell of a smear because of the minimum shrinkage and distortion in such cells.

- Smears permit a better evaluation of the nature of inflammation or infection.
- Special stains can always be used as they are used in tissue sections.
- Changes due to irradiation and other forms of therapy are easily evaluated.

LIMITATIONS OF PAP SMEAR

- The interpretation of the morphological cellular changes is based mainly on individual observation and often cannot be forced into rigid criteria. This interpretation is sometimes subjective.
- The cytologic diagnosis is not always final. It must often be confirmed by histopathology.
- The cytologist bases the diagnosis on the study of minute cellular details, while the histopathologist mainly examines the tissue pattern.
- The location of the lesion cannot be pinpointed by cytology.
- The interrelation and arrangement of the cells cannot be established.
- Neighboring cell in a smear often originate from different parts of the organ.

- The relation of the cells to the supporting stroma which is important in the diagnosis of an invasive carcinoma compared with carcinoma in situ cannot be determined by cytology.
- The size of the lesion cannot be appreciated by cytology because the number of exfoliated cells has little correlation to the size of the lesion.
- The exfoliated cells may not represent the true nature of the lesion. Poorly differentiated cells for example are the only cells exfoliating from neoplasm with mixed components.
- The screening of a smear can be time consuming and often the nature of the lesion is not obvious as in a histopathological section. The type of the lesion like in situ as compared with early invasion, adenocarcinoma or sarcoma is more difficult to determine by a smear.

ADVANTAGES OF AgNOR STAINING

- Single step modified colloidal silver technique is easy to do than the cumbersome procedure of pap stain which involves a lot of chemicals.
- It can be used on archival as well as fresh material.
- It can be observed in smears, squash cytology and histopathological sections.
- It does not require any special fixative or complex apparatus.

LIMITATIONS OF AgNOR STAINING

- The counting procedures adopted are manual and hence long and tedious. Observer error is the major cause of inaccuracy and inconsistency.
- The dots of AgNOR interphase nuclei need not always correspond actively to the number of such types in the karyotype as found by Underwood and Giri 1998[35].
- Overlap and coalescence may result in misjudged counts as mentioned by Crocker J et al[26]. The chemicals used in AgNOR stain are costly when compared to that of pap stain.

MATERIAL AND METHODS

The present study has been carried out in the Department of Pathology, Madurai Medical College and Madurai, India for a period of 2 years from June 2005 to May 2007.

The cytological materials were obtained in the form of smears which were fixed in 95% alcohol for pap and AgNOR staining . Details of the patients such as age at marriage, parity, contraception and symptoms were recorded in the working proforma which is appended in the annexure 1.

PAP STANING:

Materials required:

- Harris hematoxylin was prepared using potassium alum and mercuric oxide and filtered into a dark bottle for storage. The working solution was replaced every 1 to 3 weeks, depending on the number of slides being stained.

- OG 6

Orange G 1.0% solution in 95% Alcohol - 100ml.

Phosphotungstic acid - 0.015gm.

- EA 36

Light green SF yellowish - 0.14% in 95% alcohol	-	45ml.
Bismark brown Y-0.5% in 95% Alcohol	-	10ml.
Eosin yellow -0.55% in 95% alcohol	-	45ml.
Phosphotungstic acid	-	0.2 gm.
Lithium carbonate, saturated aqueous solution	-	1 drop.

PROCEDURE

1. Slides were transferred directly from the fixative, without drying, to 95% alcohol, and brought down through 70 and 50% alcohols to distilled water.
2. Slides were stained in Harris hematoxylin for 5 minutes.
3. And gently rinsed briefly in distilled water.
4. They were dipped in 0.25%Hcl in 50% ethanol (acid alcohol) about six times for 20 seconds.
5. And placed in running tap water for 6 minutes.

6. They were rinsed in distilled water and run through 70% 80% to 95% alcohol.

7. And stained in OG 6 for 3 minutes..

8. Rinsed in two changes of 95% alcohol.

9. Stained in EA 36 for 3 minutes.

10. Rinsed in three changes of 95% alcohol.

Dehydrated in absolute alcohol, followed by equal parts of absolute alcohol and xylol, cleared in xylol and mounted. The smears were analyzed and the cellular details were evaluated under light microscopy. The results were entered in the working proforma in the annexure -1 under The Bethesda System and WHO formulation.

Each of the samples was then subjected to an argyrophilic staining for the nucleolar organizer region according to the modified colloidal silver technique of Crocker et al[26].

AgNOR STAIN

PREPARATION OF STOCK SOLUTION
SOLUTION A

1ml of formic acid was dissolved in 99ml of distilled water and a 1% solution of formic acid was made. Two grams of gelatin powder was dissolved in

100ml of 1% formic acid by agitation and warming up to 60 degree centigrade. This comprised stock reducing solution.

PREPARATION OF SILVER SOLUTION

SOLUTION B

A 50% solution of silver nitrate was prepared by dissolving 2gm of crystalline silver nitrate in 4 ml of distilled water.

PREPARATION OF THE SILVER COLLOIDAL STAINING SOLUTIONS

4ml of freshly prepared solution B was mixed with 2ml of the stock of solution A and immediately used.

STANING PROCEDURE

1. The smears were layered with 50% silver nitrate in 2% gelatin formic acid solution.
2. The slides were then incubated in dark room for 40 minutes at room temperature.
3. The slides were then washed in distilled water dehydrated cleared and mounted in D.P.X. mountant.

AgNOR SCORE

The stained slides were viewed under oil immersion and intra nuclear silver dots are hand counted making use of light microscope. After counting at least 100 cells, AgNOR score was calculated; i.e., mean number of AgNOR dots per nucleus. The count was repeated by another person to minimize observer error.

Three main types of AgNOR configuration could be described in normal or neoplastic cells. They are depicted in the diagrams 1 to 3.

In diagram 1, the NORs are fully aggregated to form a solitary, rounded argyrophil structure, often called an AgNOR but corresponding to the nucleolus.

Diagram 2 shows a nuclear pattern which is often seen in proliferating cells where NORs can be seen within the nucleolus.

Diagram 1: TWO RESTING LYMPHOCYTES SHOWING ONE OR TWO NUCLEOLI

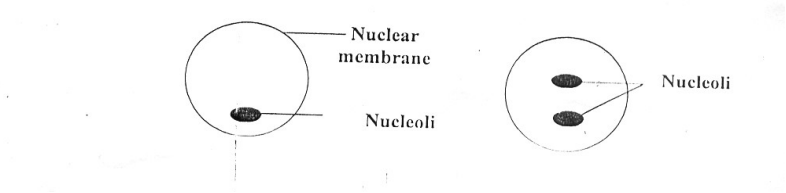


Diagram 2: A TUMOR CELL SHOWING AgNORs WITHIN THE NUCLEOLI

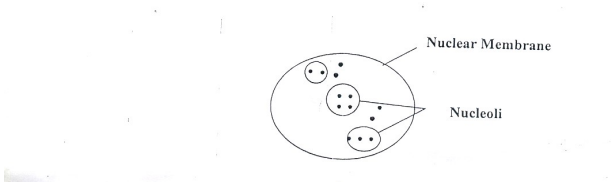


Diagram 3: A MALIGNANT CELL SHOWING AgNORs LYING FREE IN THE NUCLEUS

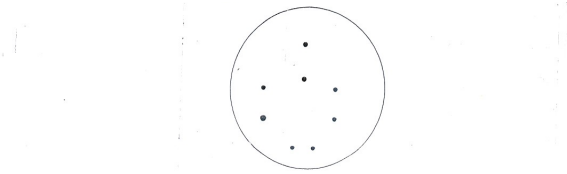


Diagram 3 represents the distribution of small true AgNORs throughout the nucleoplasm as frequently observed in highly malignant cells[26].

There are two approaches to count AgNORs:

Firstly all silver stained structures could be counted but when laying in groups; each cluster was treated as one structure. [Type 1 method]

Secondly, where AgNORs can be separately seen within a nucleolus, each AgNOR could be counted as a unit, together with the smaller AgNORs seen outside the nucleolus [type 2 method]. A type 2 method had been followed in our study.

It had been observed that type 2 method of counting have higher AgNOR scores than type 1 method of counting. The mean AgNOR count per nucleus was also graded for each case.

HISTOPATHOLOGY

The histopathology specimens of cervix biopsy were fixed in 10% formalin. The tissue slices were processed, paraffin, blocked, 5 microns thin sections were cut and stained by Hematoxylin and Eosin as described by Bancroft in Theory and Practice of Histological Techniques[37]. The unstained slides of tissue section were immersed in distilled water for 10 minutes, deparaffinised and AgNOR stain was done on those slides as described earlier and AgNOR count was done.

The results of histopathological study of H and E stained sections and AgNOR count of AgNOR stained sections were entered in the proforma.

The information collected regarding all the selected cases were recorded in a Master Chart. Data analysis was done with the help of computer using Epidemiological Information Package (EPI 2002).

Using this software, frequencies, and percentage, range, mean, standard deviation, x^2 and 'p' values were calculated. A 'p' value less than 0.05 is taken to denote significant relationship.

Sensitivity, specificity, accuracy, positive predictive value and negative predictive values were calculated using the following formulae and taking HPE findings as the Gold standard.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + false negative}} \times 100$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{False positive + true negative}} \times 100$$

$$\text{Accuracy} = \frac{\text{True positive + True negative}}{\text{Total number of cases}} \times 100$$

$$\text{Positive predictive value} = \frac{\text{True positive}}{\text{True positive + False positive}} \times 100$$

$$\text{Negative predictive value} = \frac{\text{True negative}}{\text{True negative + False negative}} \times 100$$

Photomicrographs of the smears and sections were taken wherever needed.

PHOTO MICROGRAPHS

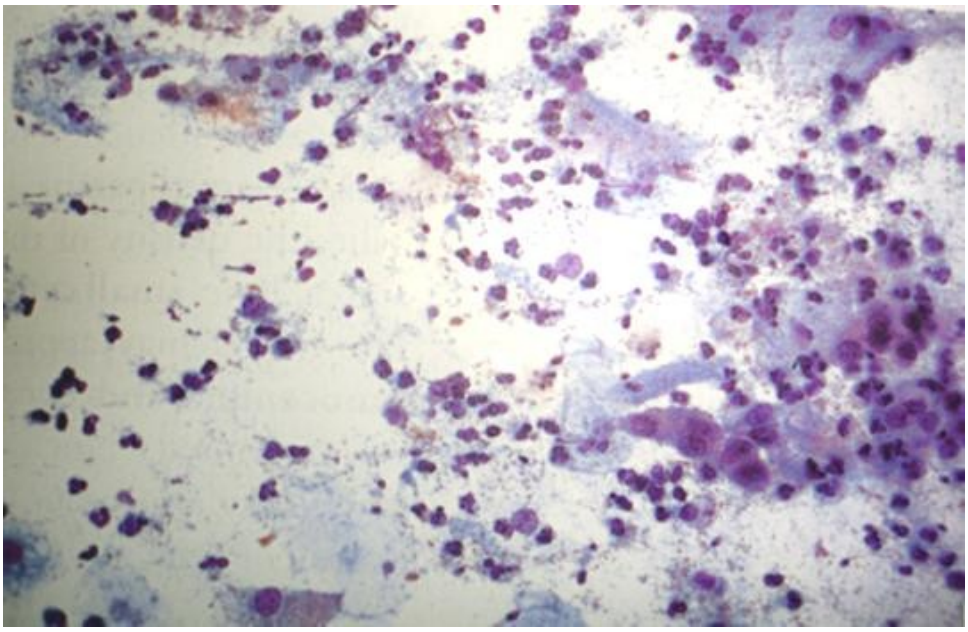


Fig 1.Polymorpho nuclear leukocytes, squamous cells and cluster of degenerated basal cells in reactive smear, Pap stain x 100 (785/05)

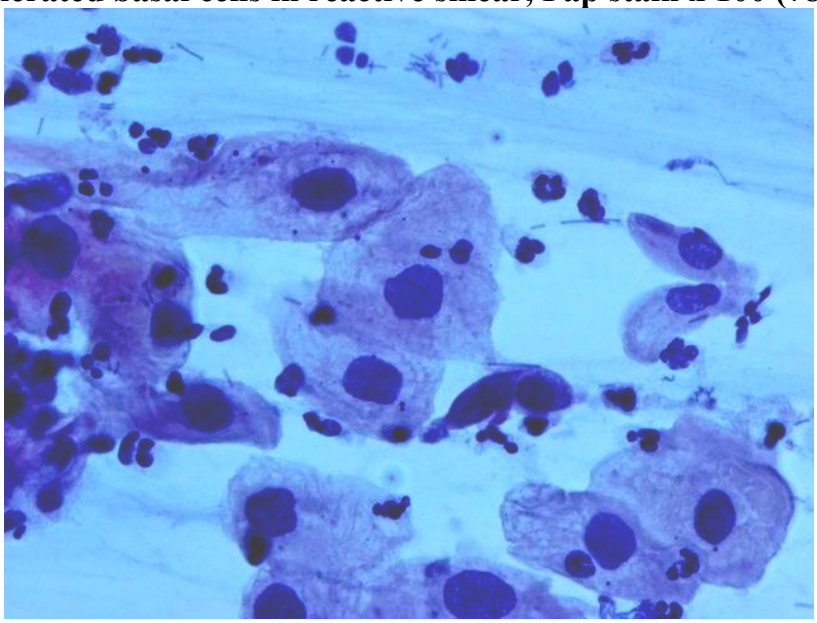


Fig 2ASCUS ,Cells show nuclear enlargement and even chromatin with superficial type cytoplasm , Pap stain x 450 (2020/05)

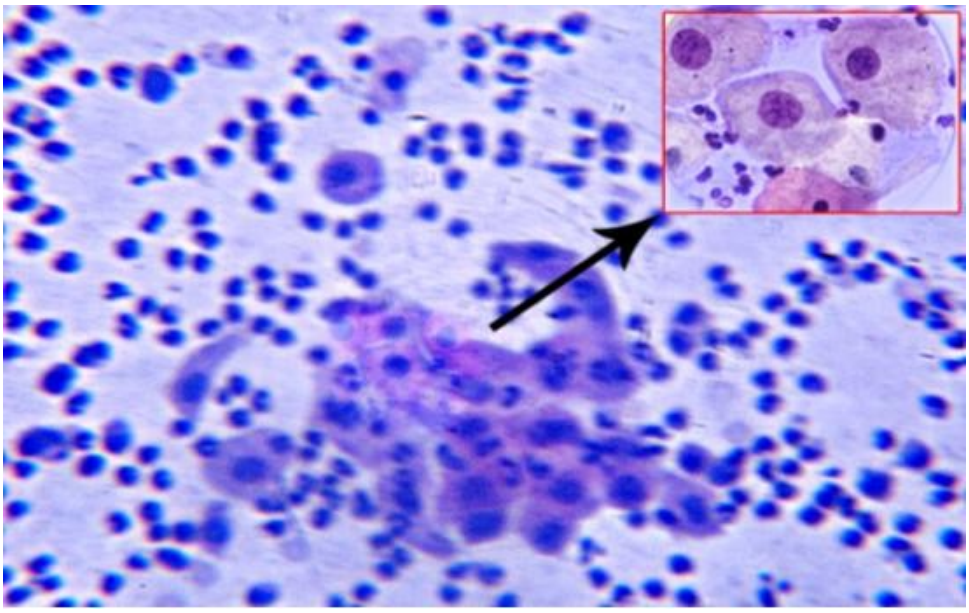


Fig 3.LSIL cells with mature intermediate type cytoplasm, fine nuclear chromatin and well defined angular cell border, Pap stain x 100 (739/06)

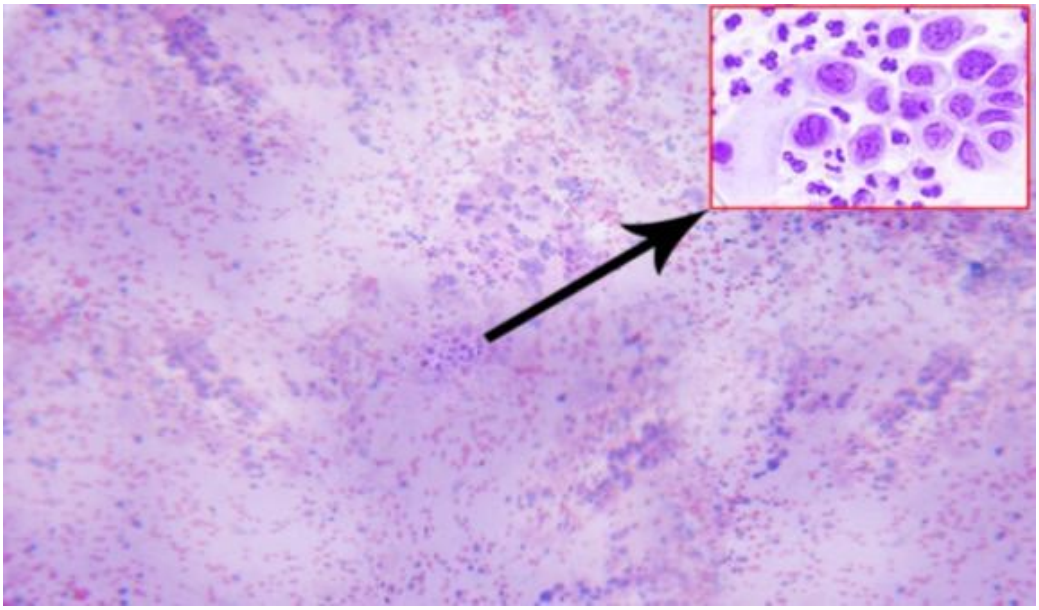


Fig 4.HSIL cells with high N/C ratio, irregular nuclear outline and nuclear chromatin distribution. Pap stain x 100 (1990/06)

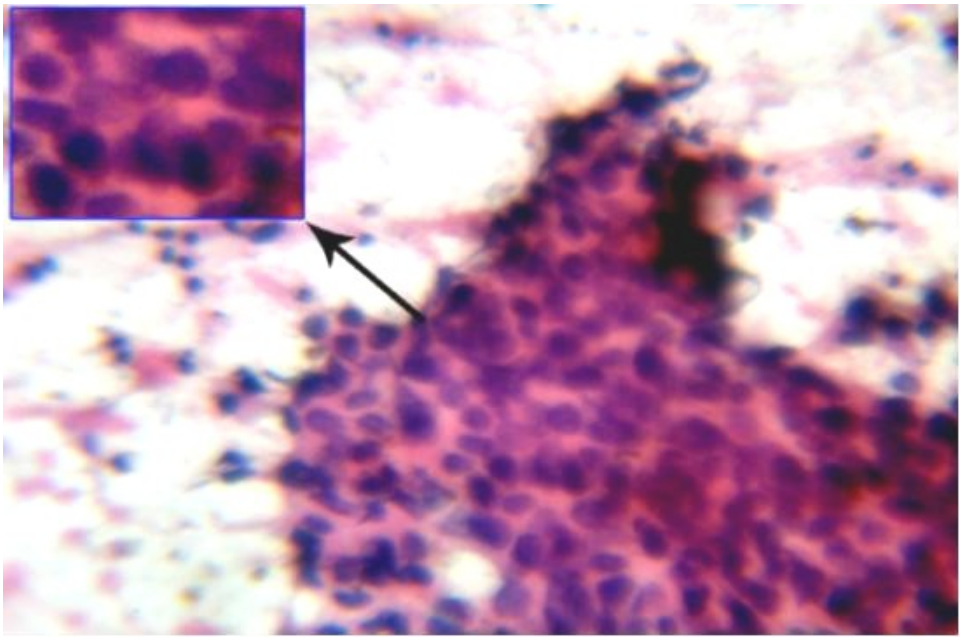


Fig 5.Malignant cell clusters with cells having indistinct cell boundaries and large irregular hyperchromatic nuclei, Pap stain x 100 (1387/06)

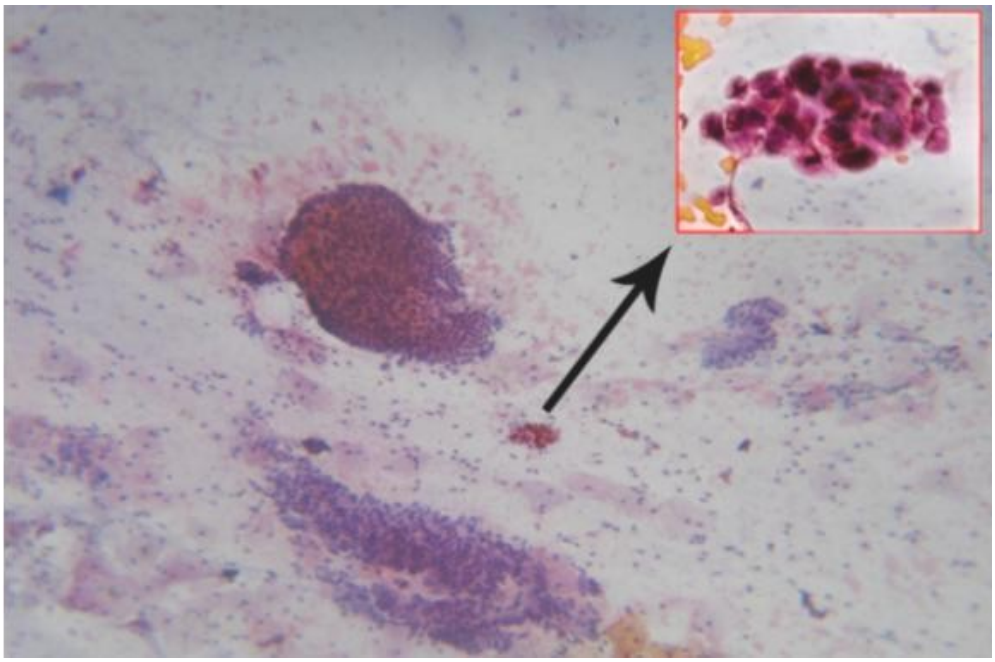


Fig 6. Cluster of malignant cells of adeno carcinoma, Pap stain x 100 (1619/06)

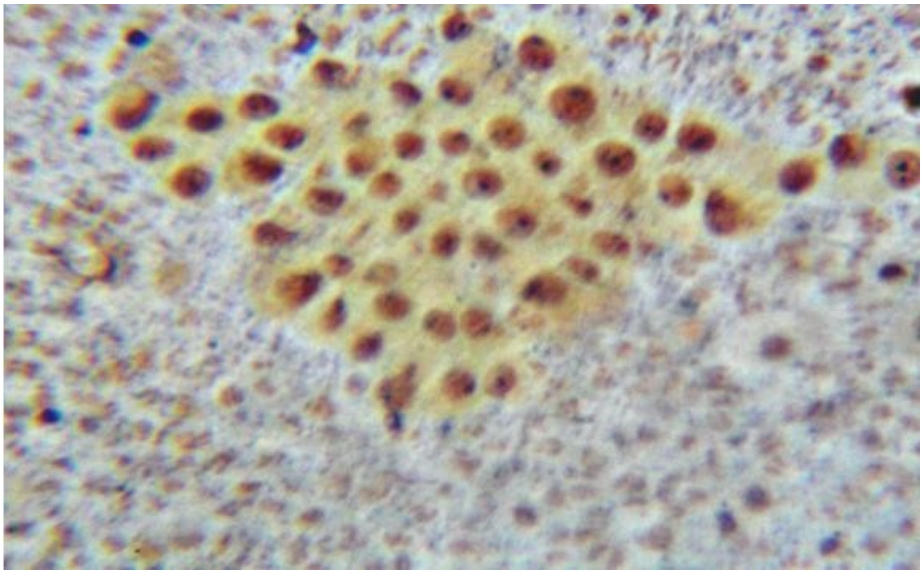


Fig 7. Ag NOR count of 3.7 in a reactive smear by TBS, AgNOR stain X 1000 (1273/05)

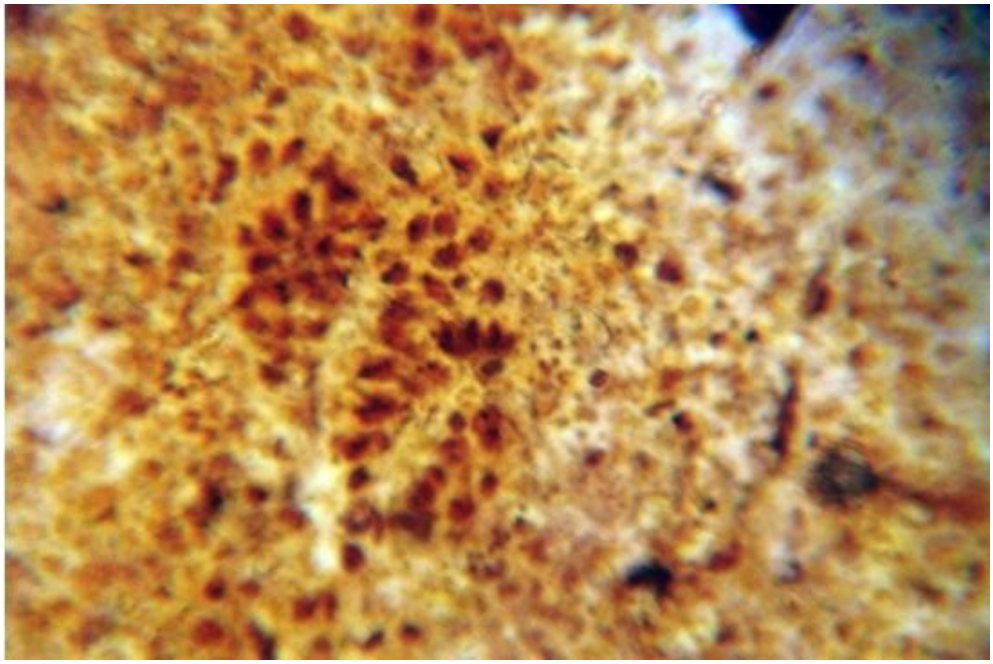


Fig 8. AgNOR count of 4.8 in a smear which showed LSIL in TBS. AgNOR stain x 1000 (1043/05)

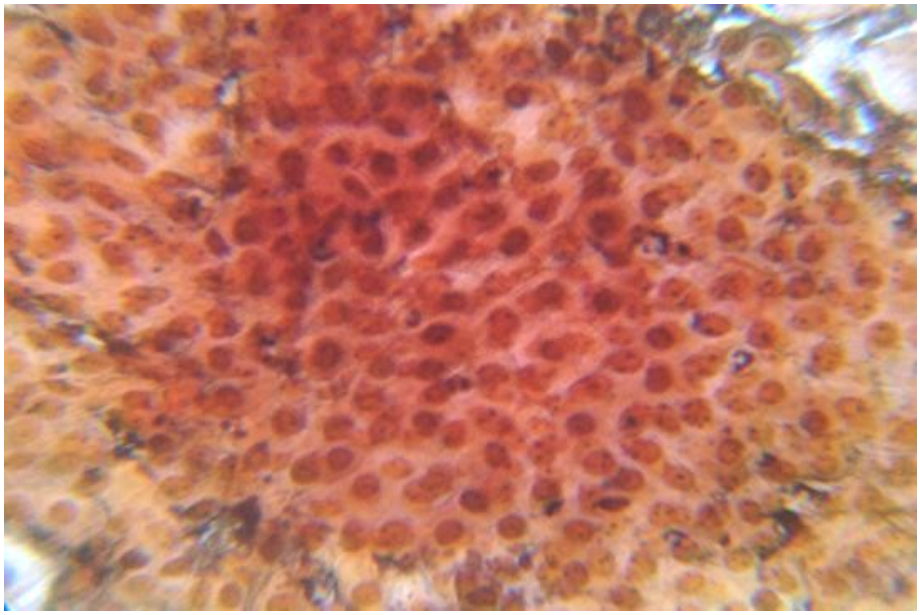


Fig 9. AgNOR count of 5.8 in a malignant cluster. AgNOR stain X 1000 (1780/05)

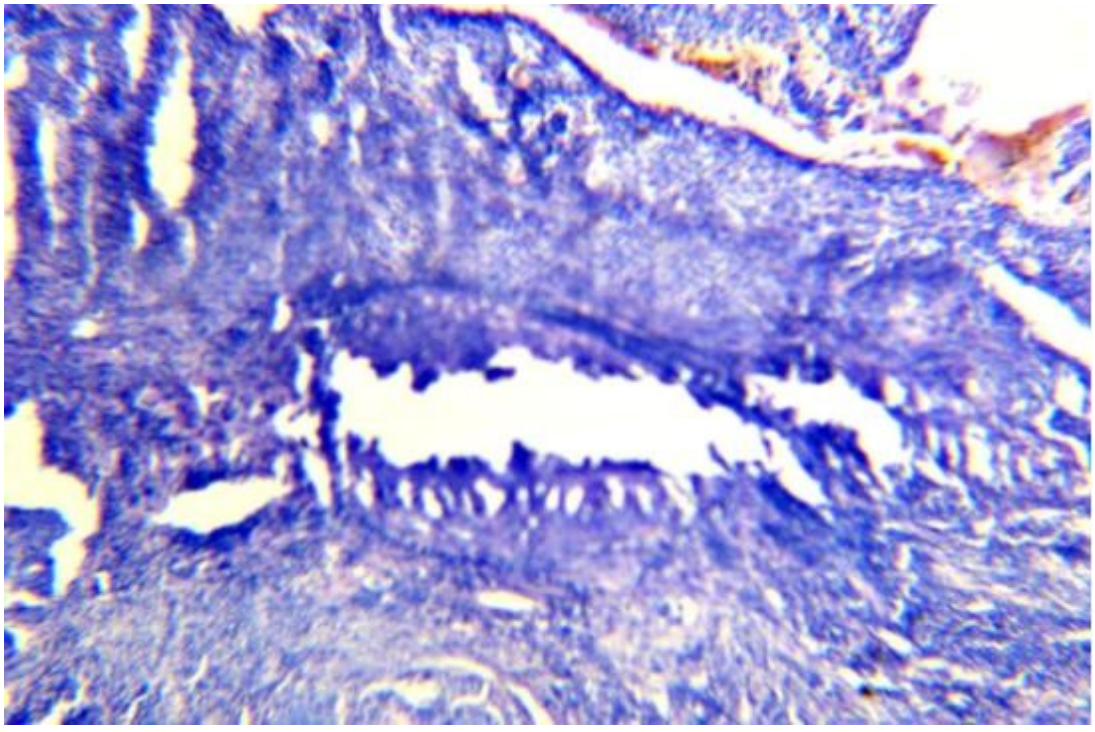


Fig 10. Chronic cervicitis with inflammatory infiltrate in the stroma, H&E stain x 450 (G2193/05)

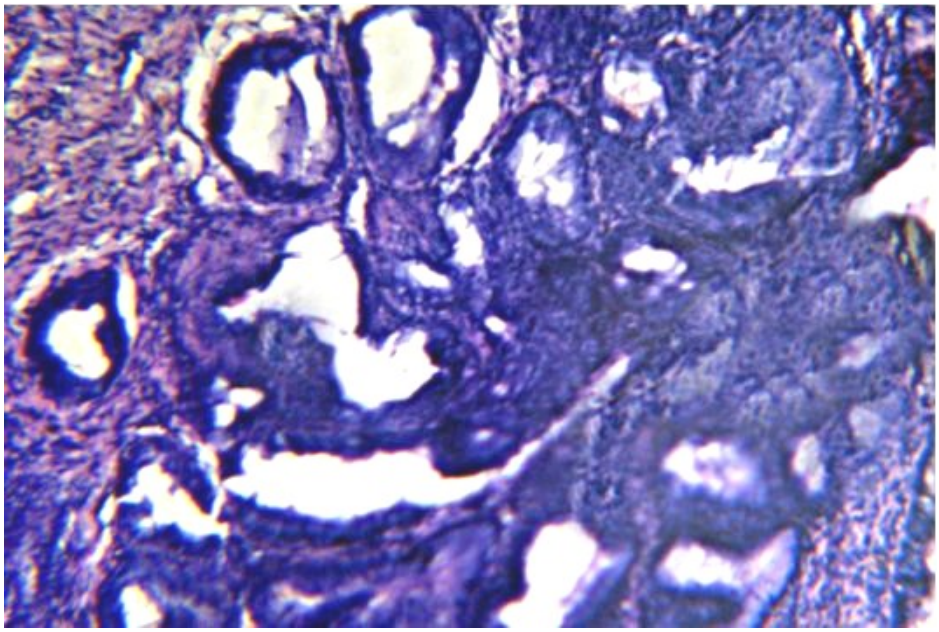


Fig 11. Squamous metaplasia. Columnar epithelium of endocervix replaced by squamous epithelium H&E stain x 450 (3298/05)

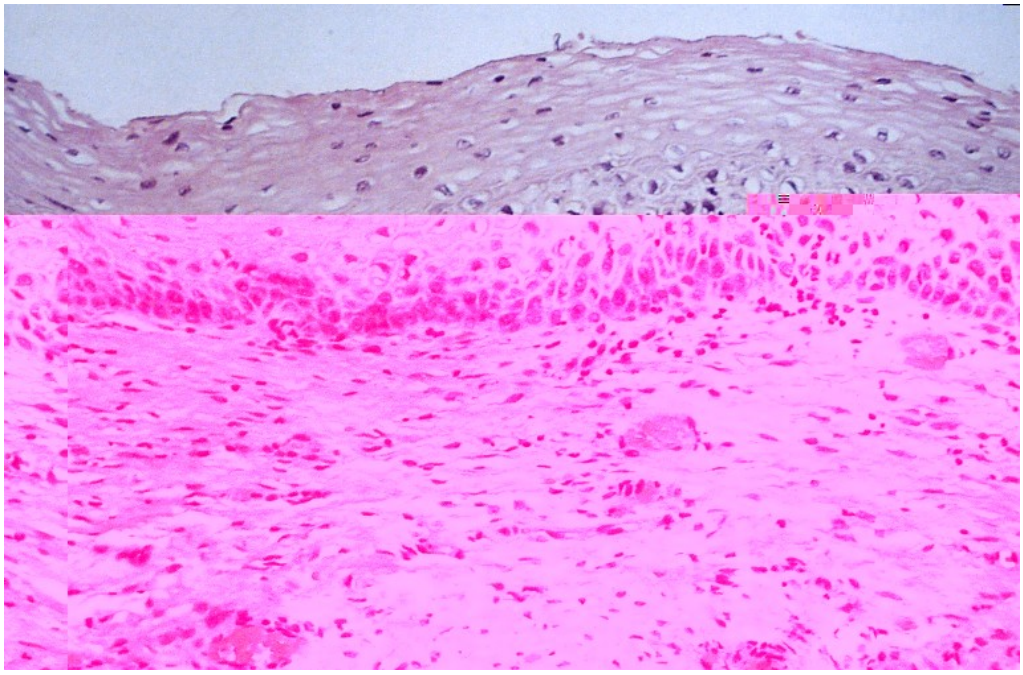


Fig12 Mild dysplasia with koilocytic changes H&E stain x 450 (G4472/06)

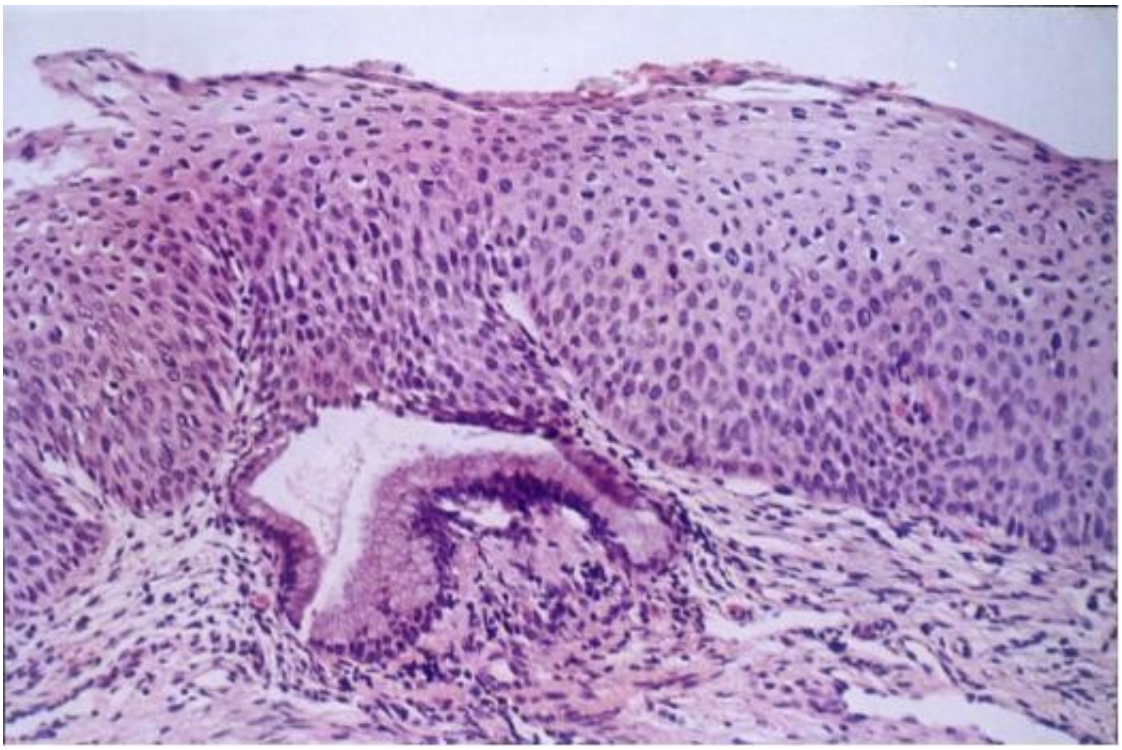


Fig 13. Moderate dysplasia, altered cell arrangement and nuclear changes limited to lower 2/3, H&E stain X 450 (G1938/05)

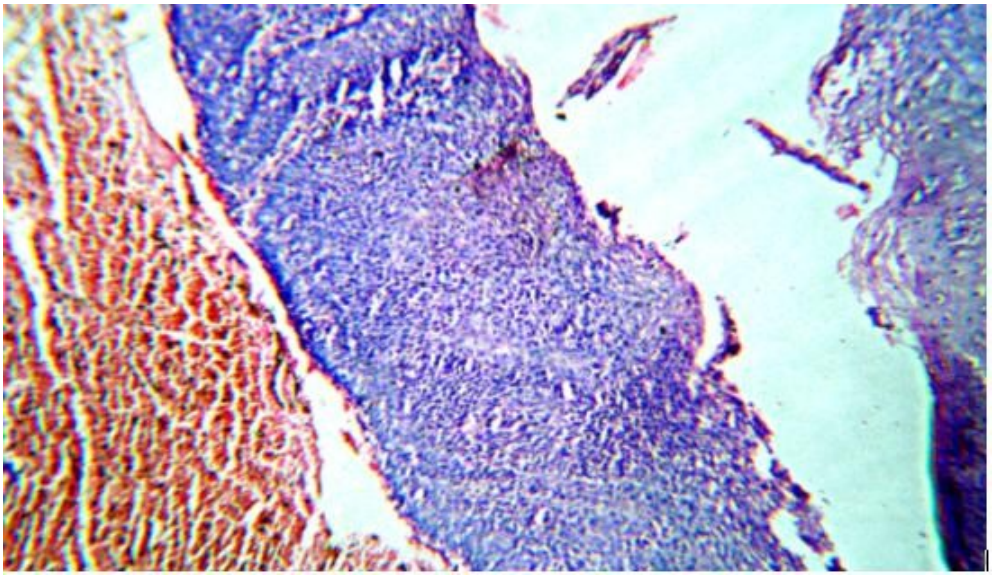


Fig 14. Carcinoma in situ, all the layers of epithelium involved, H&E Stain X 450 (G931/05)

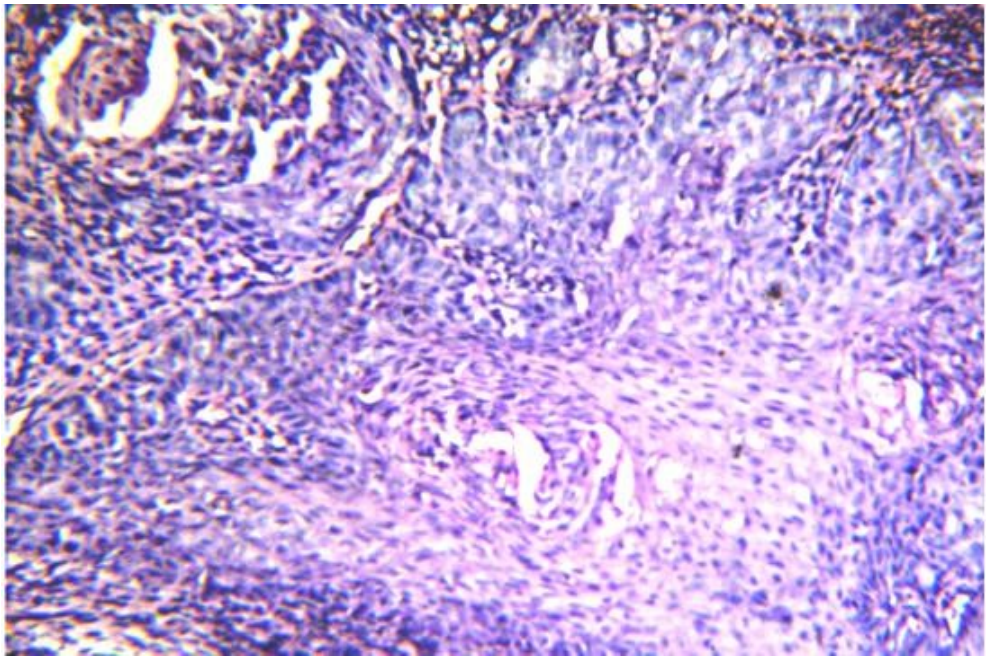


Fig15. Well differentiated SCC showing malignant squamous cells in sheets with keratinisation, H&E stain x 450 (2963/05)

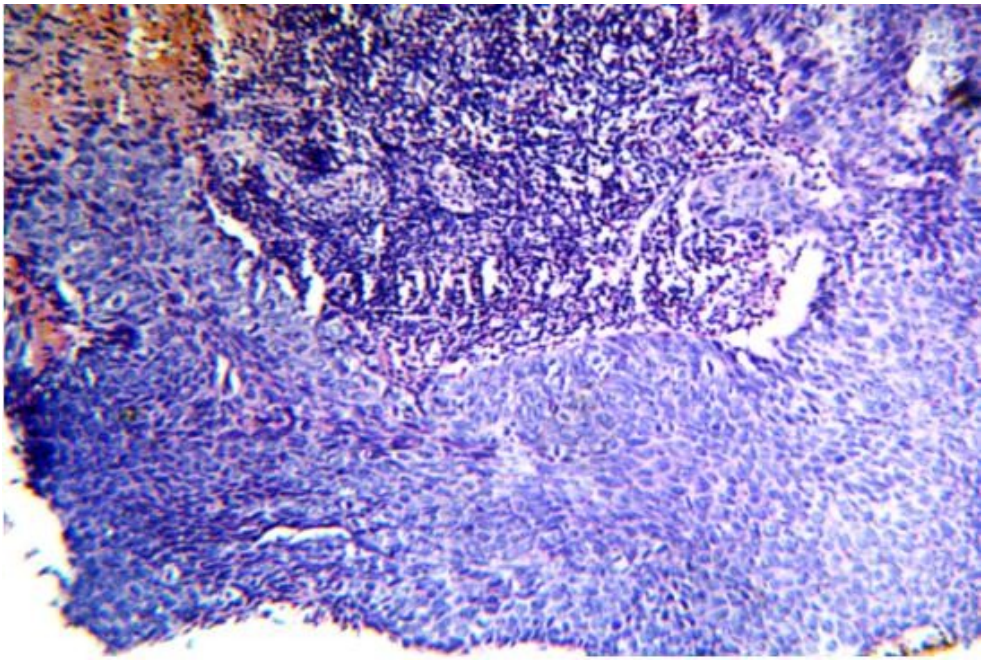


Fig16. Moderately differentiated SCC with malignant squamous cells in Sheets, H&E stain X 45 (G4495/06)

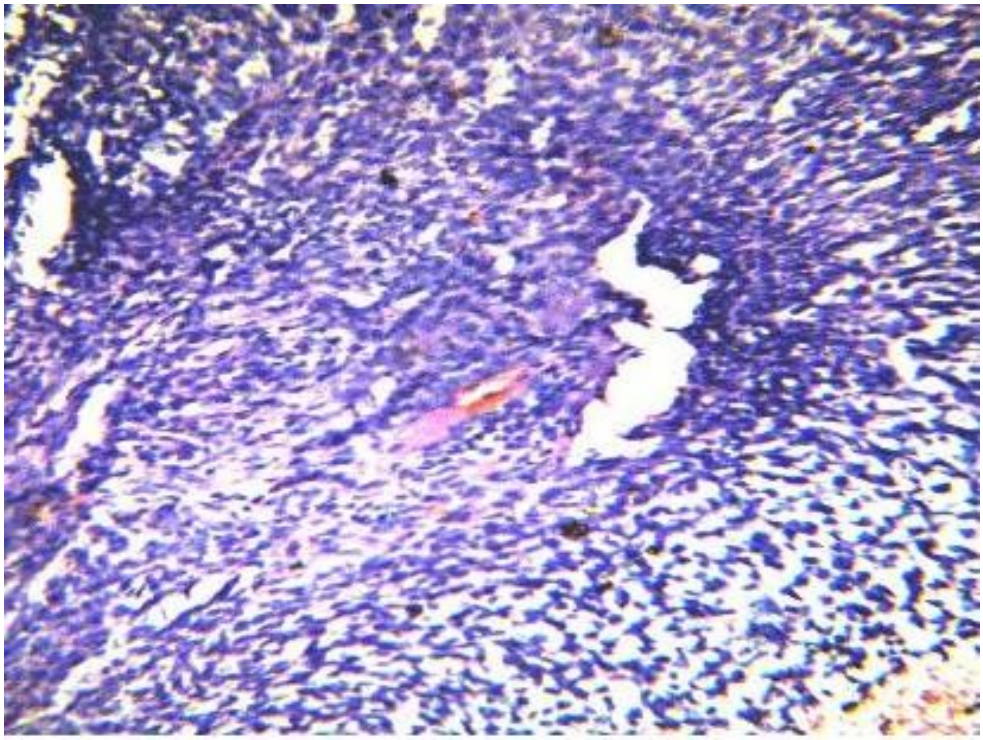


Fig 17.Poorly differentiated SCC.H&E stain x 450(G121/06)

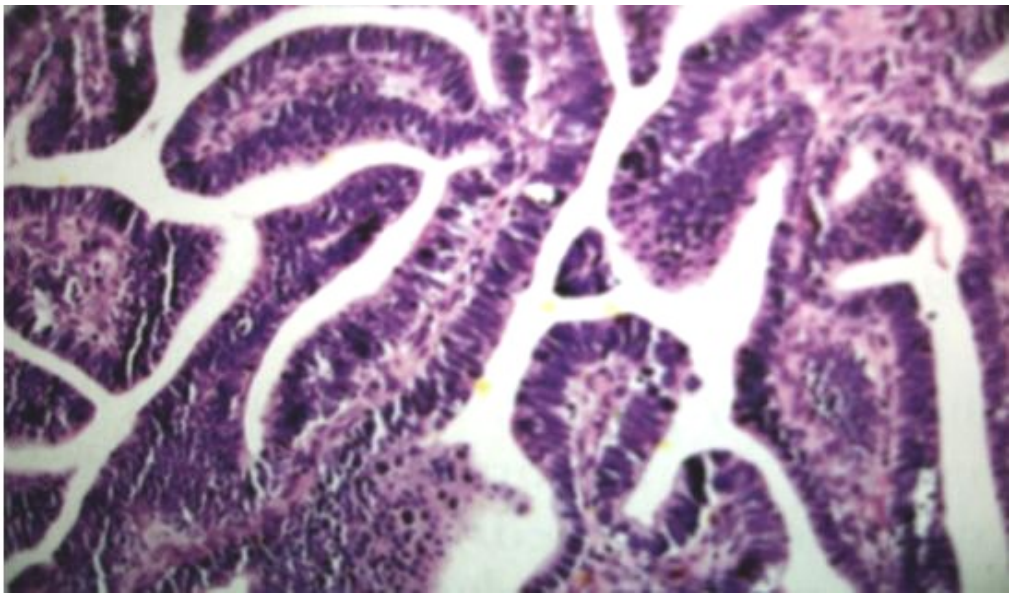


Fig 18. Adenocarcinoma with glandular pattern, H&E stain, X 450 (G 4111/06)

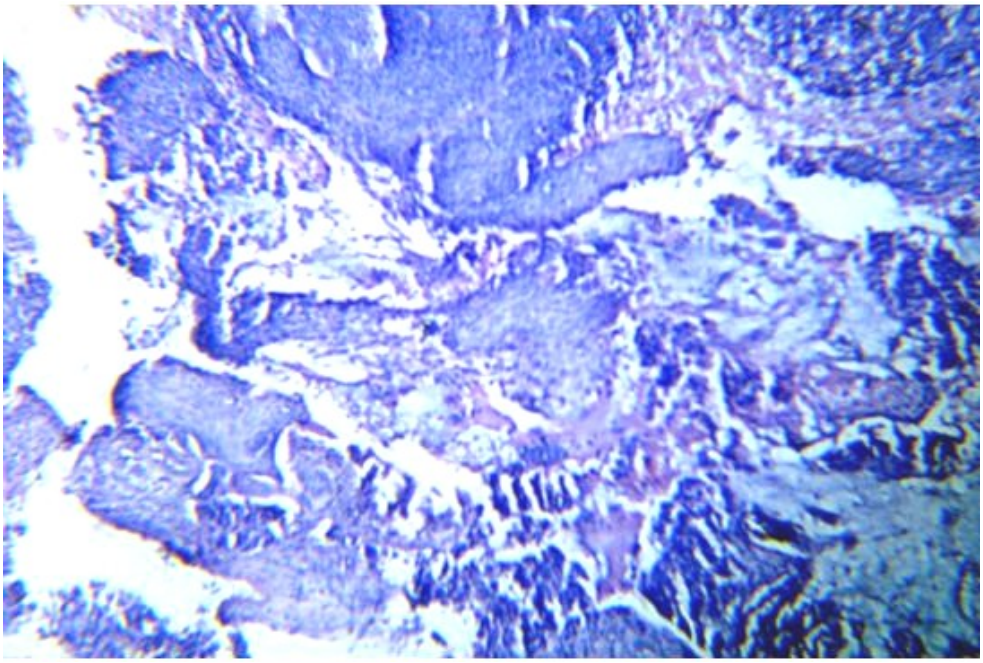


Fig 19. Malignant squamous cells in sheets with glandular elements- Adenosquamous carcinoma, H&E stain x 450 (G2846/05)

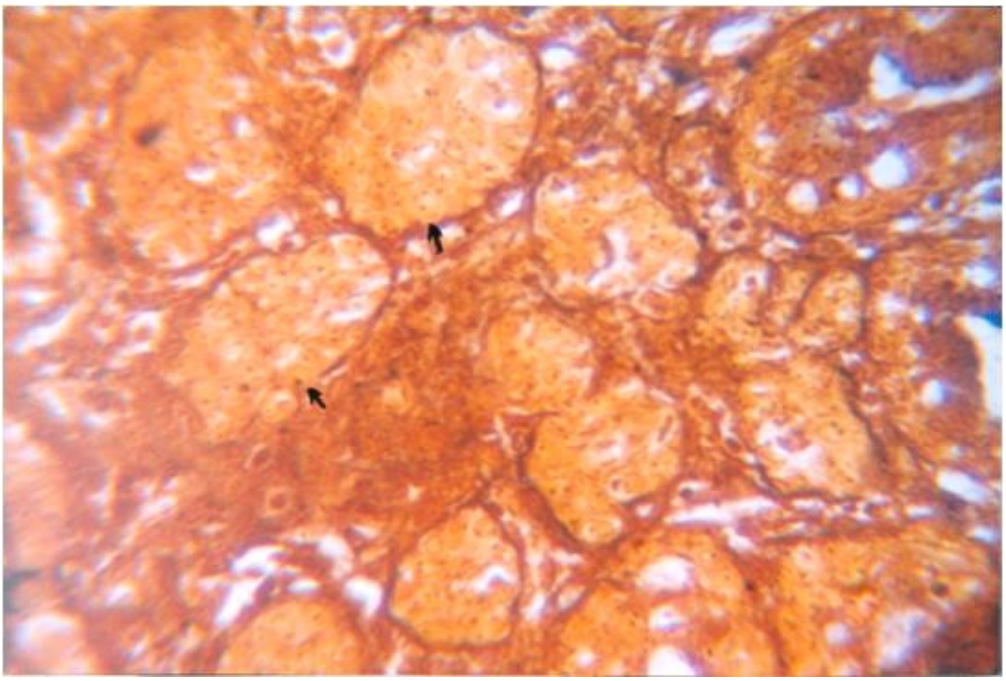


Fig 20. Pattern of AgNOR staining in squamous metaplasia, AgNOR Stain X 450 (G3298/05)

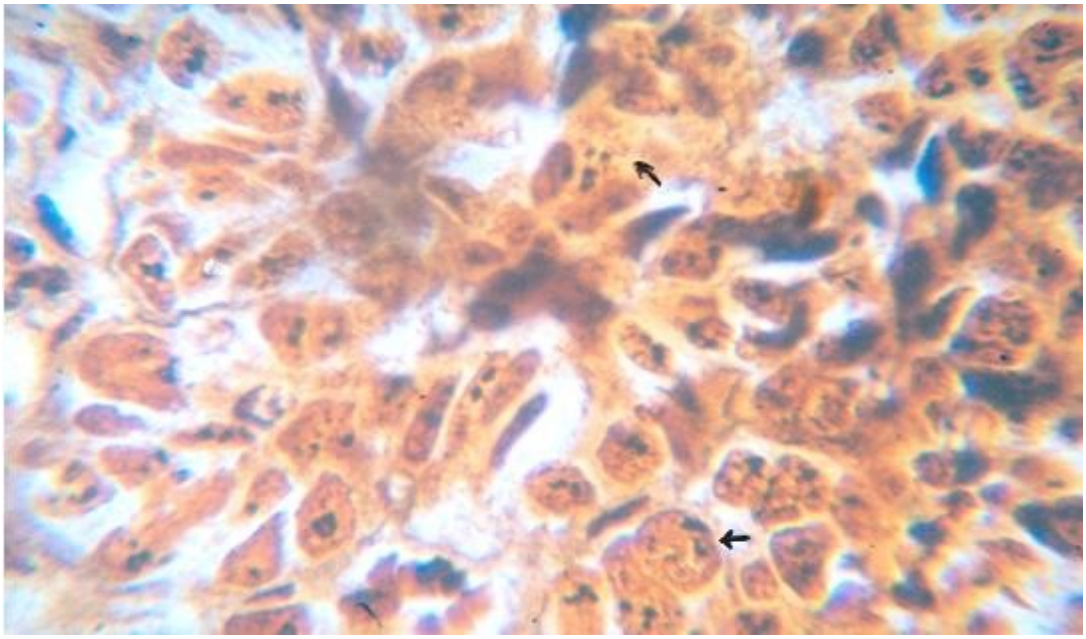


Fig 21. AgNOR pattern in carcinoma in situ, AgNOR stain x1000,(G841/05)

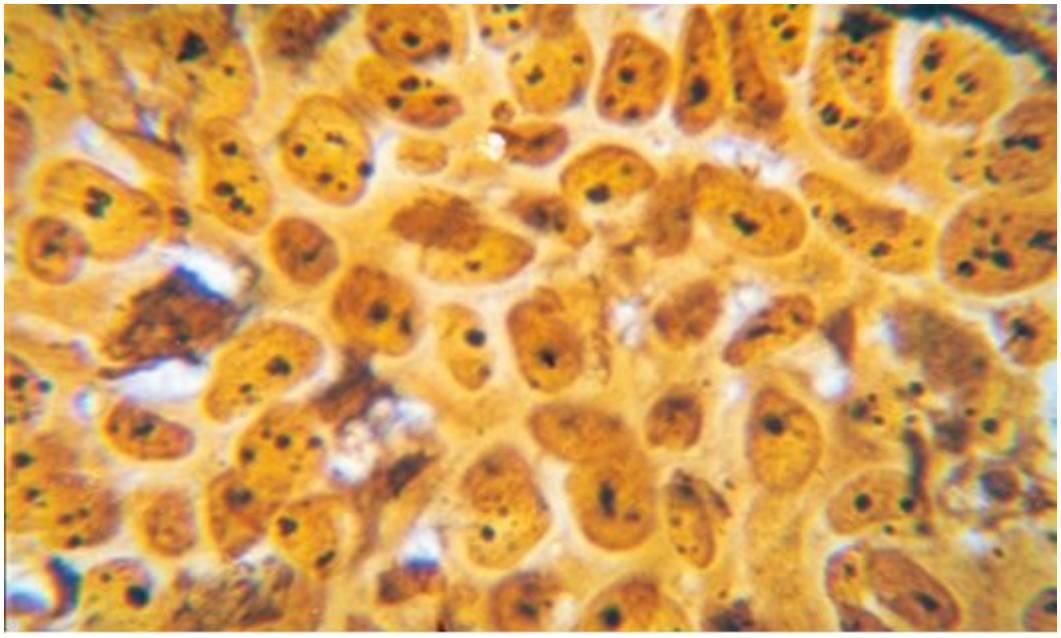


Fig 22. AgNOR count 6 in SCC, AgNOR stain x 1000(4455/06)

OBSERVATOIN AND RESULTS

In the two year study period from June 2005 to May 2007, 3842 pap smears were received from Government Rajaji Hospital, Madurai. Among these smears, 220 abnormal smears were identified and they were categorized under The Bethesda System.

Out of 220 smears studied, reactive cellular changes were 80 (36.4%).Atypical squamous cells of undetermined significance were 53 (24.1%).Low grade squamous intra epithelial lesions were only 2 (0.9%).

High grade squamous intra epithelial lesions were 33 (15%). Squamous cell carcinoma was 44 (20%). Atypical endocervical cells were only 1 (0.5%). Atypical endocervical cells favour neoplastic were 5 (2.3%) and endocervical adenocarcinoma in situ were 2 (0.9%). [Table 1 and Diagram 4]

The histopathological examination of biopsy cervix results of the above smears were compared and analysed. In the histopathological examination results, non neoplastic lesions were 97 (44.1%), pre malignant lesions were 40 (18.2%) and malignant lesions were 83 (37.7%).

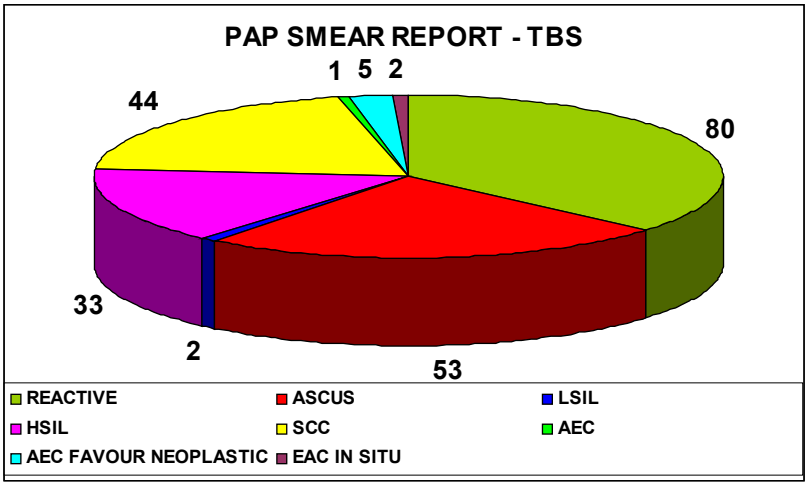
Among these 97 non neoplastic lesions, 91 (94%) were chronic cervicitis. One (1%) was endocervical polyp, 4(4%) were metaplasia and one (1%) was TB cervicitis.

TABLE 1

PAP SMEAR REPORT: TBS

PAP Smear report	No.	%
Reactive cellular changes	80	36.4
Atypical squamous cells of undetermined significance (ASCUS)	53	24.1
Low grade squamous intra epithelial lesions (LSIL)	2	0.9
High grade squamous intra epithelial lesions (HSIL)	33	15
Squamous cell carcinoma (SCC)	44	20
Atypical endocervical cells (AEC)	1	0.5
Atypical endocervical cells favour neoplastic	5	2.3
Endocervical adeno carcinoma in situ (EAC in situ)	2	0.9
Total	220	100

DIAGRAM - 4



Among 40 premalignant lesions, 14 (35%) mild dysplasia, one (3%) moderate dysplasia and 25 (62%) severe dysplasia with carcinoma in situ were detected.

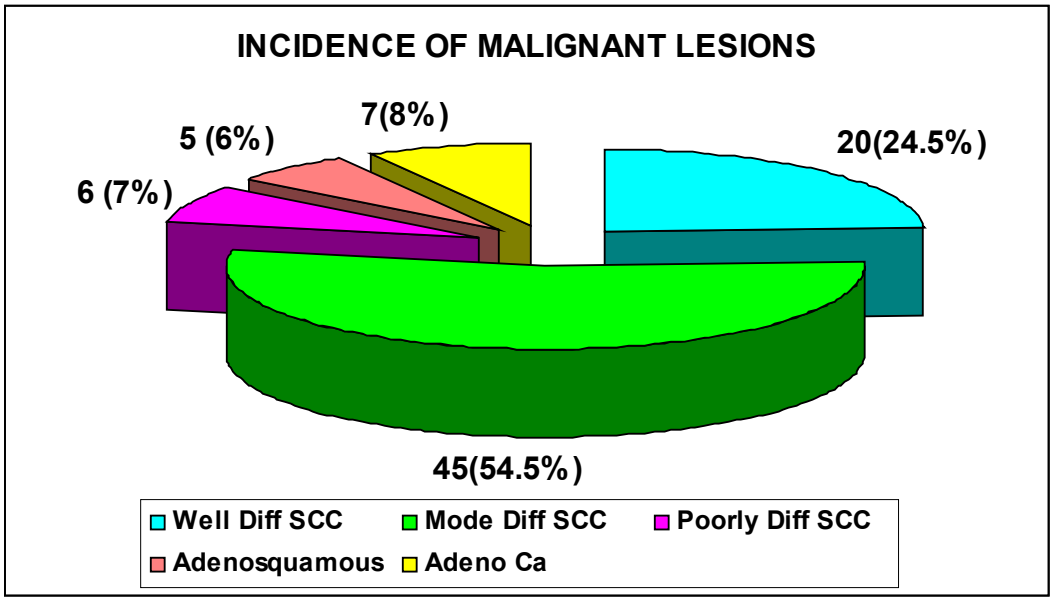
The total number of malignant lesions was 83. Out of these, well differentiated squamous cell carcinoma were 20 (24.5%), moderately differentiated SCC were 45 (54.5%) poorly differentiated SCC were 6 (7%) adenosquamous were 5 (6%) and adenocarcinoma 7 (8%) [Table 2 and Diagram 5].

TABLE – 2

INCIDENCE OF MALIGNANT LESIONS

HPE results	Number	Percentage
Well Differentiated SCC	20	24.5
Moderately Differentiated SCC	45	54.5
Poorly Differentiated SCC	6	7
Adenosquamous Carcinoma	5	6
Adeno Carcinoma	7	8
Total	**83**	**100**

DIAGRAM - 5



AGE INCIDENCE

Age group started from 21 upto 75 was included in the study. Age between 21 to 30 years were 33(15%), 31 to 40 years were 66(30%), 41 to 50

years were 69(31.4%), 51 to 60 years were 34(15.5%) and more than 60 years were 18(8.2%) [Table 3].

AGE AT MARRIAGE

In our study, cases with age at marriage 18 and below 18 were 148 (673) and between 19-30 were 72 (32.7) [Table 4]

PARITY

In this study, nulliparous women were 2(0.9%), para 1 were 7(3.2%), para 2 were 78(35.5%), para 3 and above were 133(60.4%) [Table 5]

SYMPTOMS

164(74.5%) cases presented with white discharge whereas 52(23.6%) presented with bleeding p/v. And only 4 cases (1.9%) presented with mass descending p/v. So the commonest and early symptom was excessive white discharge p/v [Table 6].

TABLE 3

AGE

Age Group (in years)	No.	%
≤ 20	-	-

21-30	33	15
31-40	66	30
41-50	69	31.4
51-60	34	15.5
>60	18	8.2
Total	220	100
Mean	44.1	
S.D.	11.5	

TABLE - 4

AGE AT MARRIAGE

Age at marriage (in years)	**No.**	**%**
≤ 18	148	67.3
19-30	72	32.7
Above 30	-	-
Total	220	100
Mean	18.8 yrs	
S.D.	4.0 yrs	

TABLE 5

PARITY

Parity	**No.**	**%**
Nulliparus	2	0.9
1	7	3.2
2	78	35.5
3 & >3	133	60.4
Total	220	100

TABLE 6

SYMPTOMS

Symptoms	No.	%
White discharge	164	74.5
Bleeding p/v	52	23.6
Mass descending p/v	4	1.9
Total	220	100

CYTOLOGICAL EVALUTION OF PAP SMEARS REPORTS

During this study period, 220 abnormal adequate smears with cellular changes were received. They were stained, evaluated and categorized under TBS.

Under TBS, 80 (36.4%) were with reactive cellular changes. The cells had cyanophilic cytoplasm and round to oval nucleus with fine chromatin in the background of degenerated basal cells and polymorpho nuclear leukocytes. (Fig.1).

ASCUS cases were 53(24.1%) The criteria for ASCUS were the nuclear enlargement with evenly distributed chromatin and superficial or intermediate type cytoplasm (Fig 2).

LSIL were 2 (0.09%) with enlarged hyperchromatic nucleus, intermediate type cytoplasm and well defined cell borders in cytology (Fig 3) and HSIL were 33 (15%) in which there were increased number of cells with high nuclear cytoplasmic ratio, irregular nuclear outline and nuclear chromatin distribution (Fig 4).

44 (20%) cases were SCC, in which cells were arranged as syncytial masses. The cells had indistinct cell boundaries with large irregular hyperchromatic nuclei. (Fig 5) one case (0.5%) of atypical endocervical cells, 5 (2.3%) cases of atypical endocervical cells favour neoplastic. There are 2 cases (0.9%) of adenocarcinoma in situ in cytology with pallisading of columnar tumour cells (Fig 6).

WHO grading was done for 136 smears. WHO grade I were 50 (36.7%), WHO grade II were 2 (1.5%) and WHO grade III and above were 84 (61.8%).

When the AgNOR dots on pap smears were analysed, 2.1 to 3 were 29 (13.2%) and 3.1 to 4 were 71 (32.3%), 4.1 to 5 were 54(24.5%), 5.1 to 6 were 26 (11.8%), 6.1 to 7 were 22(10%) and more than 7 were 18(8.3%).

In this study, mean AgNOR score for reactive lesions was 3.1 (Fig7), ASCUS was 4.6 LSIL was 4.8 (Fig 8), HSIL was 4.9, SCC was 5.8 (Fig 9) atypical endocervical cells was 4.8, atypical endocervical cells favour neoplastic

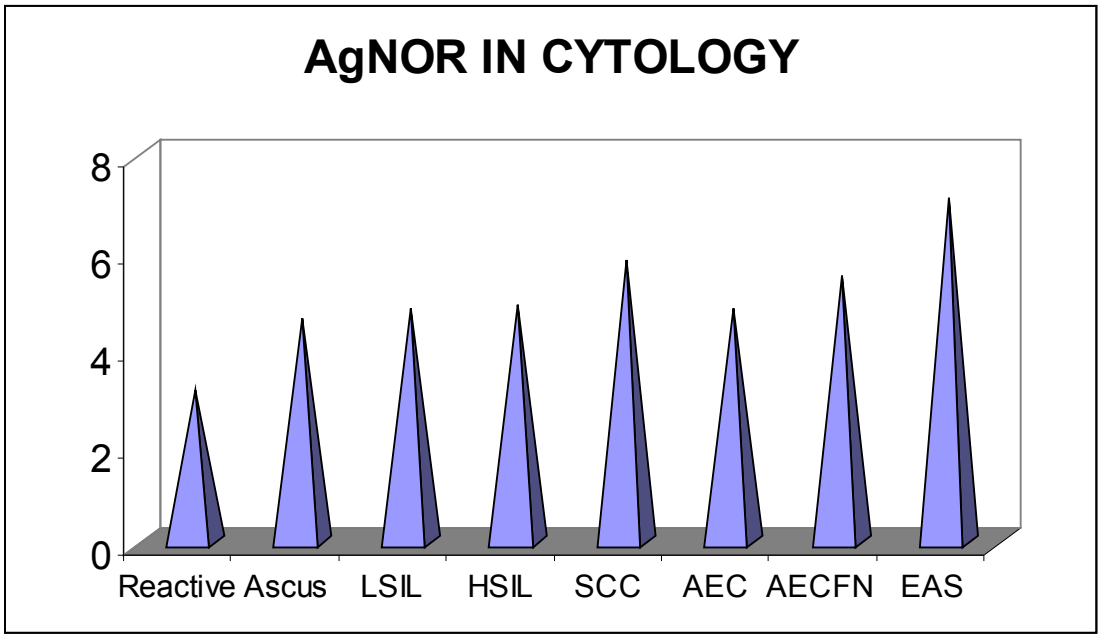
was 5.5 and endocervical adenocarcinoma in situ is 7.1. Malignant cases have higher AgNOR than reactive cases [Table 7 and Diagram 6].

TABLE 7

AgNOR IN CYTOLOGY

PAP Smear results	AGNOR - PAP
	Mean
Reactive cellular changes	3.1
ASCUS	4.6
LSIL	4.8
HSIL	4.9
Squamous cell carcinoma	5.8
Atypical endocervical cells	4.8
Atypical endocervical cells favour neoplastic	5.5
Endocervical adeno carcinoma in situ	7.1

DIAGRAM – 6



HISTOPATHOLOGICAL DIAGNOSIS

Out of 220 cases, 91cases (41.4%) belonged to chronic cervicitis with inflammatory infiltrate in the stroma (Fig 10). One case was endocervical polyp (0.5%) and one(0.5%) was tuberculous cervicitis. 4 cases (1.8%) were squamous metaplasia in which the endocervical columnar epithelium was replaced by squamous epithelium.(Fig. 11) 14(6.4%) cases were mild dysplasia in which the nuclear changes were limited to the basal layers of epithelium (Fig 12) and 1(0.5%) case was moderate dysplasia with cellular nuclear changes limited to two thirds of thickness of epithelium. (Fig 13) Severe dysplasia with carcinoma in situ were 25 (11.4%) where all the layers were affected by abnormal tumour cells with intact basement membrane (Fig. 14) well differentiated SCC with

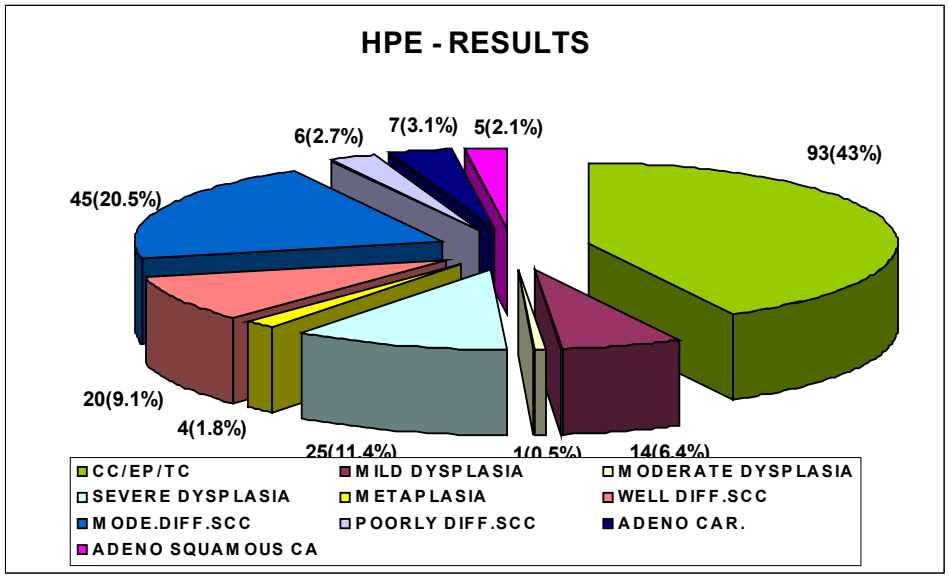
keratinization (Fig. 15) were 20 (9.1%), moderately differentiated SCC with malignant squamous cells in sheets (Fig.16) were 45 (20.5%), poorly differentiated SCC (Fig. 17) were 6 (2.7%), adenocarcinoma (Fig. 18) were 7(3.1%), adenosquamous carcinoma with malignant squamous cells in sheets and glandular elements (Fig. 19) were 5(2.1%). [Table -8 and Diagram – 7].

TABLE 8

HPE RESULTS

HPE Results	No.	%
Chronic cervicitis	91	41.4
Endocervical polyp	1	0.5
Tuberculous cervicitis	1	0.5
Metaplasia	4	1.8
Mild dysplasia	14	6.4
Moderate dysplasia	1	0.5
Severe dysplasia with carcinoma in situ	25	11.4
Well differentiated SCC	20	9.1
Moderately differentiated SCC	45	20.5
Poorly differentiated SCC	6	2.7
Adeno carcinoma	7	3.1
Adeno squamous carcinoma	5	2.1
Total	220	100

DIAGRAM - 7



When the AgNOR dots were analysed in silver colloidal solution stained 220 histopathological sections, the AgNOR count of 1.1 to 2 category were 3(1.4%) and 2.1 to 3 were 24 (10.9%), 3.1 to 4 were 64(29.10%). 4.1 to 5 were 42 (19.1%). 5.1 to 6 were 28(12.7%) 6.1 to 7 were 34(15.4%) and more than 7 were 25(11.40%).

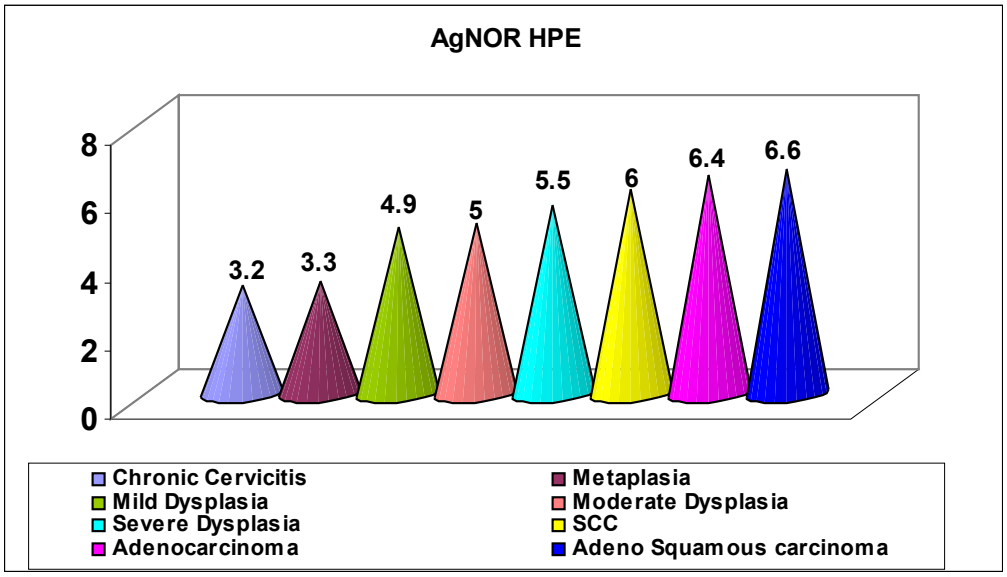
In our study there is no significant difference in AgNOR counts between squamous metaplasia (Fig 20) and chronic cervicitis The mean number of AgNORs per nucleus is significantly higher in dysplasia (mild 4.9, severe 5.5) and malignant lesions (squamous cell carcinoma 6, adeno carcinoma 6.4) as compared to metaplasia (3.3) and chronic cervicitis (3.2) [Table 9 and Diagram 8].

TABLE 9

AgNOR IN HPE

HPE results	AGNOR HPE
	Mean
Chronic cervicitis	3.2
Metaplasia	3.3
Mild dysplasia	4.9
Moderate dysplasia	5
Severe dysplasia with carcinoma in situ	5.5
SCC	6
Adeno carcinoma	6.4
Adeno squamous carcinoma	6.6

DIAGRAM - 8



All carcinomas and severe dysplasia (Fig. 21) have significantly higher AgNOR counts per nucleus compared to reactive lesions. NOR counts are higher in adenocarcinoma when compared to squamous cell carcinoma. Statistical analysis reveals significant difference between mean AgNOR counts of chronic cervicitis and dysplasia; mild dysplasia and severe dysplasia, severe dysplasia

and invasive carcinoma, squamous cell carcinoma (Fig.22) and adeno carcinoma.

Overall the mean AgNOR counts for carcinoma cervix are increased from chronic cervicitis to dysplasia.

CYTOHISTOPATHOLOGICAL CORRELATION

Out of 80 reactive cellular changes detected, 75(93.8%)cases were reported as chronic cervicitis in histopathological diagnosis, 3(3.8%) cases were squamous metaplasia, one(1.3%) case was mild dysplasia and one(1.3%) case was moderately differentiated SCC.

53 smears belonged to ASCUS category. Their histopathological follow up showed 18 (34%) chronic cervicitis, 12 (22.6%) mild dysplasia, 8 (15.1%) severe dysplasia with carcinoma in situ, 1(1.9%) squamous metaplasia, 7 (13.2) well differentiated SCC, 7(13.2%) moderately differentiated SCC. That is among 53 smears, 19 were reactive lesions, 20 were preinvasive lesions and 14 were malignant lesions.

2 smears belonged to LSIL category among which one (50%) turned out to be mild dysplasia and another (50%) severe dysplasia with carcinoma in situ.

33 smears belonged to HSIL category which in HPE showed one (3%) moderate dysplasia, 16 (48.5%)severe dysplasia with carcinoma in situ, 8

(24.2%) well differentiated SCC, 7 (21.2%) moderately differentiated SCC and one (3%) poorly differentiated SCC.

Out of 52 malignant cases detected in cytology all of them were found out to be malignant in HPE sections also.

Table 10 illustrates the correlation obtained between cytological and histopathological diagnosis.

Coming to the evaluation of efficacy of pap smear test,

True positive cases were 122,

False positive cases were 19,

True negative cases were 77,

False negative cases were 2.

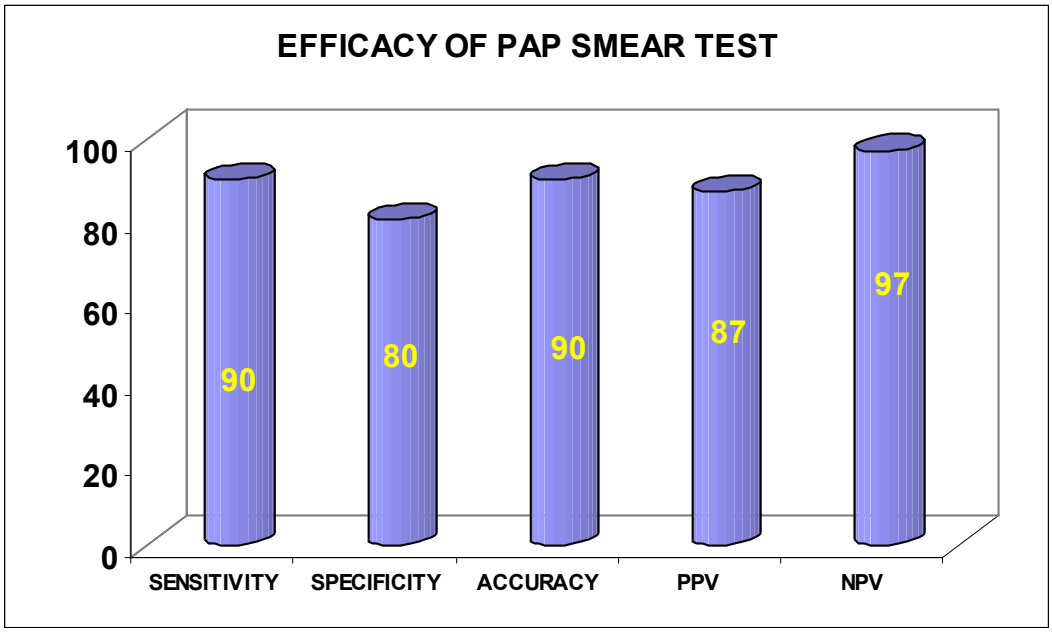
So the overall sensitivity was 90%, specificity was 80% accuracy was 90% and positive predictive value was 87 [Table 11 and Diagram 9].

TABLE 11

EFFICACY OF PAP SMEAR TEST

True positive	False positive	True negative	False negative	Sensitivity	Specificity	Accuracy	PPV	NPV
122	19	77	2	90	80	90	87	97

DIAGRAM - 9



DISCUSSION

This study is aimed at finding out the efficacy of pap smear in detecting malignant lesions of cervix in the preinvasive stage and the usefulness of the adjuvant AgNOR stain in the detection of that lesion.

AGE GROUP

In our study the age group of 21 to 30 are 33 (15%), 31 to 40 are 66 (30%) and 41 to 50 are 69 (31.4%), 51 to 60 are 34 (15.5%) and more than 60 are 18 (8.2%) with higher age incidence noted in fifth decade.

In British Columbia study 1990, the age group of 15 to 18 were 27%, 20 to 34 were 68%, 35 to 59 were 46% and 60 and above were 21% with highest age incidence noted in third and fourth decade[38].

The mean age of the patient increases as the severity of the lesion increases. The mean age of chronic cervicitis is 38.8, where as that of mild dysplasia is 40, severe dysplasia is 48 and malignant lesions are more than 47. Shalini et al showed that 32 year was the mean age of patients with benign pathology in their study.

Dysplasia was found to be more common between 20 and 39 years. Kushtagi and Fernandes in their study showed the prevalence of dysplasia was higher in women over 30 years[39].

Among malignant lesions, mean age of well differentiated SCC cases are 47.9 years, moderately differentiated SCC are 50 years and poorly differentiated SCC are 53.7 years in our study.

Squamous cell carcinoma was found to be more common between 40-and 60 years. Shalini et al showed 25% of well differentiated and 35% of moderately differentiated carcinomas in the age group 40-49, 50% of well differentiated and 57% of moderately differentiated carcinoma was in age group > 50. The mean age of patient with cancer cervix was 41[40].

AGE AT MARRIAGE

In our study, among 220 cases, 148(67.3%) are married at or below the age of 18 and 72[32.7%] cases are married between 19 to 30. So it is clear that cervical lesions are more common in early age of marriage and early age of intercourse which expose the genital tract to various pathogenic organisms like HPV, physical and chemical trauma .

Incidence of squamous cell carcinoma was about 50% in patients with marital life more than 20 years. Kushtagi et al had demonstrated that increase in severity of underlying CIN lesions occur with increase in the duration of years of marital life[39].

PARITY

Out of 220 cases, 2 are (0.9%) nulliparous while para1 are 7 (3.21%), para 2 are 78 (3.5%) para 3 are 76 (34.5%) and para more than 3 are 57 (25.9%).

The mean parity for reactive lesions is 2.9, pre malignant lesions is 2.9 and malignant lesions is 3.1 in the smear results. The mean parity for reactive lesions was 2.6 where as 3 for premalignant lesions and 3 for malignant lesions in HPE results.

Malignant cases have high parity than the other cases. So this relationship is statistically significant. Multiparity is one of the etiological factors for cervical lesions. Various family planning methods usually adopted by the women modify the status of parity which should be kept in mind when there is significantly increasing incidence of premalignant and malignant lesions in low parity groups.

In this study, there are 2 (0.9%) nulliparous women with cervical lesions. Pattern et al reported a prevalence of 0.98% cervical lesions in non gravid women[41]. A study by Shalini et al showed the mean parity was 4.2 in patients with invasive cancer. Kushtagi and Fernandez showed that the prevalence of CIN was significantly higher in parity of more than 2. This might be attributed to hormonal and nutritional changes that occur in pregnancy, immuno suppression during pregnancy and cervical trauma during vaginal delivery[42].

CYTO HISTOPATHOLOGICAL CORRELATIONS

80 cases cytologically show reactive cellular changes. In the correlative histopathological study of these 80 cases, 73(91.2%)show chronic cervicitis, one (1.25%) is tuberculous cervicitis, one (1.25%) is endocervical polyp and 3 (3.8%) with squamous metaplasia, one case (1.3%) with reactive cellular changes

show mild dyplasia and another case (1.3%) show moderately differentiated SCC [Table – 10].

53 cases belong to ASCUS category. In histopathological correlation, 18 (34%) show chronic cervicitis, 1(1.9%) show squamous metaplasia, 12(22.6%) show mild dysplasia, 8(15.1%) show severe dysplasia with carcinoma in situ, 7(13.2%) show well differentiated SCC and 7(13.2%) show moderately differentiated SCC. That is 19 reactive lesions, 20 preinvasive lesions and 14 malignant lesions are found. So we detected 8 preinvasive lesions from ASCUS category which is very beneficial to the patient regarding prognosis. Premalignant lesions need further follow up by repeat smears, colposcopy directed biopsy if available or cone biopsy.

2 cases belong to LSIL category among which one (50%) turned out to be mild dysplasia and another (50%) severe dysplasia with carcinoma in situ.

Among 220 cases, 33 are HSIL category. Their histopathological correlative study show one (3%) moderate dysplasia, 16(48.5%) severe dysplasia with carcinoma in situ, 8(24.2%) well differentiated SCC,7(21.2%) moderately differentiated SCC and 1(3%) poorly differentiated SCC.

44 out of 220 smears are SCC category. In HPE, 5(11.4%) well differentiated SCC, 30(68.2%) moderately differentiated SCC 4(9.1%) poorly differentiated SCC and 5 (11.4%) adenosquamous carcinoma. In all cases of histopathologically proved malignancies, smears showed positivity for malignancy.

Only one smear show atypical endocervical cells which turned out to be adeno carcinoma in HPE. Atypical endocervical cells favour neoplastic is seen in 5 smears and all of them are histopathologically proved to be adenocarcinoma. There are 2 cases of adenocarcinoma in situ in cytology in which one of them is adenocarcinoma and another one is adenosquamous carcinoma.

Coming to the efficacy of Pap smear, the sensitivity is90%. Specificity is 80.2%and accuracy is 90%. In Jones B.A et al study sensitivity is 89.4%, specificity is 64.8% and accuracy is 88.9%[43] [Table 12].

TABLE 12

COMPARATIVE STUDY – PAP SMEAR AND HPE

	Present study **Percentage**	**Jones BA Study** **Percentage**
Sensitivity	90	89.4

Specificity	80	64.8
Accuracy	90	88.9

False negative are 2 cases which are due to error in sampling, screening or interpretation of smears. Tritz et al found discrepancies between cytologic and histologic diagnoses in 69 out of 615 (11%) patients with a cytologic diagnosis of neoplastic abnormality Source of error may be inappropriate biopsy or faulty biopsy[44].

Small size of the tumour cells and their scarcity in smears are the major sources of false negativity[45]. Adhesion of cells within the abnormal epithelium is another reason for false negativity.

There are 19 false positive cases in our study. It may be due to inadequate biopsy or misinterpretation of benign process. Anderson and Jones documented patients with abnormal smears and initial lack of confirmation by biopsy require long term follow up to discover occult neoplastic lesion[46]. False positivity may be due to removal of entire lesion by energetic brushing resulting in biopsies with denuded surface or misinterpretation of cluster of endocervical cells with large nuclei and nucleoli, cluster of endometrial cells or postmenopausal atrophic cells as abnormal cells.

AgNOR STAINING IN PAP SMEAR

In this study, mean AgNOR score for reactive lesions is 3.1 .ASCUS is 4.6, LSIL is 4.8, HSIL is 4.9, SCC is 5.8, atypicalendocervical cells is 4.8 atypical endocervical cells favour neoplastic is 5.5 and endocervical adenocarcinoma in situ is 7.1. Malignant cases have higher AgNOR than reactive cases.

In a study by J.J.Misra et al, there was a progressive increase in AgNOR count when the severity of the lesion was increased. The statistical analysis show p value<0.05 between normal and inflammatory lesions. And highly significant difference between inflammatory and LSIL cases, between LSIL and HSIL and between severe dysplasia and frankly malignant cases. ($p<0.01$) Eagan et al observed that mean AgNOR count increased steadily whereas the mean size of AgNORs decreased from CIN I to CIN II[32].

Cardillo studied AgNOR counts in cervical smears of squamous metaplasia and cervical intra epithelial neoplasia. The smears previously stained with Papanicolaou technique were destained and restained with AgNOR silver. He found statistically significant difference ($p < 0.05$) in AgNOR counts in squamous metaplasia and various grades of CIN[47].

AGNOR STAINING IN HPE

In our study there is no significant difference in AgNOR counts between

squamous metaplasia and chronic cervicitis. The mean number of AgNORs per nucleus is significantly higher in dysplasia (mild 4.9, moderate 5, severe 5.5) and malignant lesions (squamous cell carcinoma 6, adenocarcinoma 6.4) as compared to metaplasia 3.3 and chronic cervicitis 3.2. All carcinomas and severe dysplasia have significantly higher AgNOR counts per nucleus compared to mild dysplasia. NOR counts are higher in adenocarcinoma when compared to squamous cell carcinoma. Statistical analysis reveals significant difference between mean AgNOR counts of chronic cervicitis and dysplasia; mild dysplasia and severe dysplasia, severe dysplasia and invasive carcinoma, squamous cell carcinoma and adeno carcinoma.

Overall the mean AgNOR counts for carcinoma cervix are increased from chronic cervicitis and dysplasia.

An Indian study done by Prathiba and Kuruvilla (1995) on the role of AgNOR in diagnosis of premalignant and malignant lesions of the cervix, showed that mean AgNOR count progressively increased from normal to CIN I, CIN II, CIN III and invasive carcinoma. The difference between counts in CIN I and CIN II and invasive carcinoma was statistically significant[27].

Crocker et al in 1990 also showed statistically significant difference in AgNOR counts between CIN I, CIN II and CIN III[36]. The table 13 shows mean

AgNOR counts in various grades in different studies.

In Jyomita Agarwal, JK Gupta study (1997) AgNORs was counted in biopsies from 202 cases of various lesions and cervix. The mean number of AgNORs per nucleus was significantly higher in CIN (4.05±0.04) and Malignancy (5.50±0.65) as compared to squamous metaplasia (1.74±0.32) and chronic cervicitis (1.54±0.42). Adeno carcinomas had higher AgNOR counts as compared to other carcinomas. It concluded that the estimation of AgNORs can be helpful in distinguishing benign lesions from CIN and malignancy of the cervix[29].

In Rajni Kaushik, study, the mean AgNOR counts in cervical epithelium showed a progressive and statistically significant increase from normal to chronic cervicitis to CIN I, II and III ($p < 0.001$). Scores in carcinoma also exceeded that of CIN ($p < 0.05$). It concluded that this can prove to be a useful adjunct to routine histopathology to evaluate cervical lesions[34].

In our study also, it is noted that the size of AgNOR dots decrease with increase in AgNOR count. This is in accordance with the study reported by Eagan who noted an inverse relationship between AgNOR numbers and sizes, and proved that severe dysplasia could be distinguished from mild dysplasia on the basis of AgNOR size[48].

SUMMARY

The present correlative study revealed the following:

1. The peak age incidence of reactive lesions is fourth decade while premalignant and malignant lesions are fifth decade and above.

2. The earlier the age at marriage, they are more vulnerable to cervical lesions especially malignant.

3. The severity of cervical lesions increases with parity.

4. The most common finding in The Bethesda System (TBS) is reactive cellular changes accounting for 36.4% in cytology and 44.1% in histopathology.

5. When the 53 ASCUS category smears of TBS were followed up in HPE, 8 (15.1%) preinvasive lesions were detected.

6. Among the malignant lesions, the incidence of squamous cell carcinoma is high accounting for 20% in cytology and 32.1% in histopathology.

7. The overall sensitivity of Pap smear is 90%, specificity is 80% and accuracy is 90%.

8. The AgNOR count in Pap smear is higher in malignant cases than the reactive lesions in Pap smear.

9. The AgNOR count in premalignant lesions is higher than the reactive lesions in Pap smear.

10. Histopathological examination of the cervix is the gold standard against which the accuracy of Pap smear can be obtained and is found to be 90% in this study.

11. The AgNOR count in histopathological examination which reflects the behaviour of cells is higher that is 4.9 in mild dysplasia, 5.5 in severe dysplasia with carcinoma in situ in this study when compared to chronic cervicitis 3.2 and squamous metaplasia 3.3.

12. Adenocarcinoma has higher AgNOR count (6.4) than squamous cell carcinoma (6.0).

CONCLUSION

In conclusion, this correlative study of Pap smear and histopathological examination of cervix revealed the overall sensitivity of 90%, specificity of 80% and accuracy of 90%.

The false negative and false positive cases in this study can be minimized by proper sampling, screening, interpretation and further follow up study of repeat smears.

AgNOR stain and count in routine Pap smear and histopathological examination of cervix are found to be simple and useful adjuvant test particularly in the dysplastic lesions.

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ANNEXURE – I

PROFORMA

1. Name :
2. Age :
3. Address :

4. Unit :
5. OP/IP Number :
6. Cytology Number :
7. Menstrual History :
   - Menarche -
   - Menstrual cycle -
8. Marital Status :
   - Age at Marriage
9. Obstetrical history :
   - Number of children
   - Last child birth
   - Sterilized or not
   - Any other method of contraception
10. Presenting Symptoms Duration
    - White discharge –
    - Bleeding per vaginum
    - Mass decending per vaginum
    - Abdominal pain –
11. Clinical diagnosis :
12. Pap smear report :

    i) TBS

    * Unsatisfactory –

* Satisfactory -

(1) Negative for intra epithelial lesion -

Trichomonas Vaginalis
Fungus – Candia
Actionmyces
Herpes Simplex
Nonneoplastic Lesions

Reactive cellular changes
Glandular Cells
Atrophy

Endometrial cells

(2) Epithelial Cell Abnormalities

Atypical squamous cell of undetermined significance (ASC-US)
Low – grade squamous intra epithelial lesion (LSIL)
High – grade squamous intra epithelial lesion (HSIL)
Squamous cell carcinoma
Atypical end cervical cells
Atypical endometrial cells
Atypical endocervical cells favour neoplastic

(ii) BY C.I.N. (WHO) : Grade I (Mild)
Grade II (Moderate)
Grade III (Severe+ Ca in situ)
And above

13. Biopsy Report :

14. AgNOR :

Cytology :

H.P.E